PATENT

RESPONSE UNDER 37 C.F.R. 1.116 - EXPEDITED PROCEDURE - EXAMINING GROUP 1774

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Appl. No.: 11/997,227 — Confirmation No.: 4281
Applicant(s): Anderson *et al.*
Filed: July 3, 2008
Art Unit: 1774
Examiner: Christopher VanDeusen
Title: INACTIVATION OF GRAM-POSITIVE BACTERIA

Docket No.: 031749/340085
Customer No.: 00826

Mail Stop AF
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

AMENDMENT AFTER FINAL UNDER 37 C.F.R. § 1.116

Commissioner:

In response to the Final Office Action dated April 25, 2014, please amend the above-identified application as follows:

Amendments to the Claims are reflected in the listing of claims beginning on page 2 of this paper.

Remarks/Arguments begin on page 3 of this paper.

An **Appendix** containing a reference by Kawada *et al.* (*Journal of Dermatological Science* (2002) Vol. 30, pp. 129-135) is attached immediate following page 11 of this paper.

Clear-Vu Lighting, Exhibit 1020

Amendments to the Claims

1. (Currently Amended) A method for disinfecting air, contact surfaces, or materials by inactivating methicillin-resistant *Staphylococcus aureus* (MRSA) in the air, on the contact surfaces, or on the materials, said method comprising exposing the MRSA to visible light without using a photosensitiser, wherein the visible light for inactivating the ~~bacteria~~MRSA consists of wavelengths in the range of 400-420 nm, and wherein the method is performed outside of a human body, the contact surfaces or the materials are non-living, and the air, contact surfaces or materials are not exposed to a disinfecting dose of light at a wavelength above 500 nm.

Claims 2-5. (Canceled)

6. (Previously Presented) A method as claimed in claim 1 wherein the light consists of wavelength 405 nm.

Claims 7-18. (Canceled)

REMARKS/ARGUMENTS

Status of the Claims

Claims 1 and 6 remain pending in the application. Claim 1 has been amended to recite "the visible light for inactivating the MRSA" in order to provide correct antecedent basis. No new matter is added by way of this amendment, nor does this amendment raise new issues with regard to patentability of the claimed subject matter. Entry of this claim amendment into this application is respectfully requested in order to place the application in condition for allowance or in better condition for appeal.

Reconsideration of the claims is respectfully requested in view of the following remarks. Any new arguments addressed herein were not previously presented as Applicants believed that the previously pending claims were allowable in view of the arguments of record. The Examiner's rejections in the Final Office Action are addressed below in the order set forth therein.

The Rejections of the Claims Under 35 U.S.C. § 103 Should Be Withdrawn

Claims 1 and 6 were rejected under 35 U.S.C. § 103 as being unpatentable over Jones *et al.* (U.S. Application Publication No. 2005/0055070) in view of Burnie *et al.* (U.S. Patent No. 6,627,730) as evidenced by Bek-Thomsen *et al.* (*Journal of Clinical Microbiology* (2008) Vol. 46, No. 10, pages 3355-3360). This rejection of the claims is respectfully traversed.

Applicants' claimed invention is drawn to a method for disinfecting air, contact surfaces, or materials by inactivating methicillin-resistant Staphylococcus aureus (MRSA) using visible blue light consisting of wavelengths in the range of 400-420 nm (claim 1), or visible blue light at a specific wavelength of 405 nm (claim 6). Applicants' claimed methods also require **(i)** that the methods are performed outside of a human body, **(ii)** that the contact surfaces or materials that are disinfected are non-living, and **(iii)** that the air, contact surfaces, or materials are not exposed to a disinfecting dose of light at a wavelength above 500 nm.

Jones *et al.* teaches a method and device for the treatment of skin conditions, particularly Acne Vulgaris. The method of Jones *et al.* utilizes visible light to activate bacterial porphyrins in

the skin, using preferred ranges of 580 to 590 nm or blue light in the range of 390 to 420 nm (see, paragraph [0029]). Burnie *et al.* teaches a staphylococcal ABC transporter. The Office Action cites this reference as teaching that both MRSA bacteria and coagulase-negative staphylococcus (CNS) bacteria are drug-resistant species and that new therapies are needed for both. Bek-Thomsen *et al.* teaches that acne is not associated with yet-uncultured bacteria. The Office Action alleges that this reference provides evidence that *Staphylococcus epidermis* contributes to the symptoms of Acne Vulgaris and, therefore, could be disinfected by the method of Jones *et al.*

The Office Action asserts that the presently claimed methods are obvious in view of these cited prior art references. Applicants respectfully disagree for at least the reasons previously made of record and those further set forth below.

A. There Was No Expectation of Success That the Claimed Methods Would Be Effective Against MRSA – Results Were Not Predictable

The Office Action asserts that the cited prior art references provide an expectation of success that the method of Jones *et al.* could be used to achieve the claimed invention by disinfecting methicillin-resistant *Staphylococcus aureus* (MRSA) bacteria. The Office Action further asserts that such an outcome would be predictable in view of the cited prior art. Applicants respectfully disagree with these conclusions. On the contrary, Applicants provide in the attached Appendix a prior art reference by Kawada *et al.* (*Journal of Dermatological Science* (2002) Vol. 30, pp. 129-135) demonstrating that disinfection of MRSA bacteria using visible blue light would *not have been predictable* and that there would have been *no expectation of success*.

Kawada *et al.* describes phototherapy with a high-intensity, enhanced, narrow-band, blue light source for the treatment of acne. This reference teaches a study wherein patients were treated with visible blue light in the range of 407 to 420 nm to inactivate bacteria in the skin. This range of blue light wavelengths is within the range taught by Jones *et al.* (*i.e.*, 390 to 420 nm). As described in the section titled "3.4. Bacterial isolates," and shown in Table 2, bacterial

samples from 24 patients were obtained, cultured, and identified (see, page 133). Applicants note that certain *Staphylococcal* species of bacteria are commensal and would be expected to be found on the skin of any patient. As such, their presence on the skin of acne patients does not indicate that they contribute to acne symptoms. Rather, it is evident from its "Introduction" that Kawada *et al.* is primarily concerned with *Propionibacterium acnes* as the causative species for acne.

Notably, one patient sample was shown to be positive for *Propionibacterium acnes*, *Staphylococcus epidermis*, and _MRSA_ bacteria. With regard to this patient, Kawada *et al.* states:

> Patients who had MSSA [methicillin-sensitive *Staphylococcus aureus*] and MRSA _*discontinued the treatment*_ with the complaint of _*'worsened'*_ that was also confirmed by the investigator to be worsened [emphasis added]. (page 133)

Thus, Kawada *et al.* teaches that treatment with visible blue light in the range of 407 to 420 nm was _*not effective*_ in treating a patient that was co-infected with MRSA bacteria. Rather, phototherapy worsened the patient's condition to the degree that treatment was discontinued. Kawada *et al.* goes on to conclude that:

> Two patients in our study who showed MSSA or MRSA co-cultured with *P. acnes* and *S. epidermis* discontinued the study because of _*ineffectiveness of phototherapy*_. The main pathogen in acne lesions of these patients may have been _*S. aureus that did not respond to blue light*_ [emphasis added]. (page 134)

Thus, Kawada *et al.* clearly suggests that MRSA bacteria _*were not inactivated*_ by the use of visible blue light between 407 to 420 nm.

Therefore, the state of the art at the relevant time indicates that a person of ordinary skill in the art _*would not have predicted*_ that the method of Jones *et al.* could use visible blue light to disinfect MRSA bacteria. Furthermore, the skilled artisan would have had _*no expectation of success*_, as Kawada *et al.* clearly suggests that visible blue light _*would not be effective*_ against MRSA bacteria. Accordingly, Applicants' claimed method was unpredictable in view of the prior art, and a case for obviousness cannot be made.

Furthermore, Applicants note that Kawada *et al.* does not anticipate or render the claimed invention obvious. Kawada *et al.* only uses visible blue light on MRSA bacteria that are present *in the skin* of a patient. At no point is MRSA isolated from the skin and *then* exposed to visible blue light outside of a human body on a non-living contact surface or material, as required by instant claim 1. Moreover, Kawada *et al.* does not teach or suggest that MRSA could even be disinfected with visible blue light in the range of 400 to 420 nm.

B. The Cited Prior Art Does Not Teach That the Method of Jones et al. Is Effective Against CNS Bacteria

In making a case for obviousness, the Office Action cites the method of Jones *et al.*, which is directed to the use of visible blue light in the range of 390 to 420 nm to inactivate bacteria in the skin. The Office Action acknowledges that Jones *et al.* does not teach the disinfection of MRSA, but alleges that the method of Jones *et al.* would be broadly effective against any bacteria that "contribute to the symptoms of Acne Vulgaris" (see, page 4 of the Office Action).

Bek-Thomsen *et al.* is then cited as purported evidence that *Staphylococcus epidermis*, a coagulase-negative staphylococcus (CNS) bacteria, allegedly contributes to Acne Vulgaris. The Office Action combines the teachings of these references to conclude that *Staphylococcus epidermis* would be disinfected by the method of Jones *et al.*, and relies on this interpretation of the art to formulate a case for obviousness. However, Applicants respectfully submit that this interpretation and conclusion are flawed.

On the contrary, it is evident from Kawada *et al.* that the method of Jones *et al.* would *not* be effective against *Staphylococcus epidermis* bacteria. Specifically, Kawada *et al.* describes experiments wherein *Propionibacterium acnes* bacteria and *Staphylococcus epidermis* bacteria were isolated from patients and then irradiated *in vitro* with visible blue light in the range of 407 to 420 nm (see, page 133, section titled "3.5. Bacterial effects in vitro"). As shown in Table 3, *Propionibacterium acnes* bacteria were susceptible to the effects of visible blue light treatment, with a *significant* reduction in the number of cultured bacteria after 60 minutes. However,

visible blue light treatment had *no effect on Staphylococcus epidermis bacteria*. Kawada *et al.* states at page 133:

> As shown in Table 3, *P. acnes* was decreased in number after irradiation *whereas S. epidermis was not* [emphasis added].

Thus, the Office Action's argument is flawed, as a person of ordinary skill in the art would have concluded from Kawada *et al.* that visible blue light, such as that used by Jones *et al.*, could *not* have been used to disinfect *Staphylococcus epidermis* bacteria. Accordingly, the Office Action's case for obviousness cannot be made, as it directly relies on this flawed interpretation of the prior art.

Moreover, Applicants submit Jones *et al.* is not directed to the treatment of *any* bacteria that contributes to the symptoms of Acne Vulgaris, as asserted by the Office Action. Rather, it is clear from the disclosure of Jones *et al.* that this reference is *solely* directed to the treatment of *Propionibacterium acnes* bacteria in the skin.

In support of its position, the Office Action points to paragraph [0037] of Jones *et al.*, which states that its apparatus:

> . . . may be used to treat a region of skin affected by the condition Acne Vulgaris by causing a photochemical reaction in said region that stimulates the production of free radicals that react with, *and at least partially disable or destroy, bacteria that contribute to the symptoms of Acne Vulgaris* [emphasis added].

However, Jones *et al.* *specifically defines* such contributing bacteria as *Propionibacterium acnes* bacteria throughout the specification. For example, paragraphs [0004] and [0005] describe the factors that cause Acne Vulgaris and its development, referring *only* to *Propionibacterium acnes* bacteria as being present and rapidly multiplying in clogged pores. Paragraph [0023] directly states that the method of Jones *et al.* is drawn to the disinfection of *Propionibacterium acnes* bacteria, stating:

> Advantageously, a photo-chemical reaction is caused that *disables or destroys, wholly or partially, the bacteria Propionibacterium acnes, which, as described above, is one of the causes of Acne Vulgaris* [emphasis added].

Paragraphs [0024] and [0025] also directly support this point, stating:

> The chromophore targeted is preferably porphyrin. Porphyrin is a naturally occurring substance produced by the bacteria *Propionibacterium acnes* [emphasis added]. (paragraph [0024]); and
>
> Thus, by exciting Porphyrin *in the manner outlined above* it is possible to disable or destroy the bacterium responsible for Acne Vulgaris in a pain-free, non-invasive and efficient manner [emphasis added]. (see, paragraph [0025])

Jones *et al.* further clarifies that its method is directed to *Propionibacterium acnes* bacteria at least in paragraphs [0029] and [0093] as well. Importantly, Jones *et al.* does not teach or suggest that any bacteria *other* than *Propionibacterium acnes* is involved in the development of Acne Vulgaris or is affected by its method of using visible blue light.

Therefore, when the reference is considered as a whole, it is clear that Jones *et al.* is only directed to the treatment and disinfection of *Propionibacterium acnes* bacteria in the skin. The passage of paragraph [0037] cited by the Office Action is not intended to broadly mean that the method could be used on *any* bacteria that might contribute to Acne Vulgaris. Rather, this passage simply refers to the bacteria that Jones *et al.* *defines* as contributing to Acne Vulgaris (*i.e.*, *Propionibacterium acnes*). Accordingly, the Office Action's case for obviousness cannot be made, as it directly relies on this flawed interpretation of the prior art.

C. Official Notice Is Improperly Applied

Furthermore, Applicants believe that Official Notice is improperly applied by the Office Action when making its case for obviousness. Specifically, the Office Action states that a reasonable expectation of success exists because the same bacterial vulnerability of CNS bacteria (*e.g.*, *Staphylococcus epidermis*) "*would be exploited*" when applying the method of Jones *et al.*

to MRSA bacteria (page 6 of the Office Action, emphasis added). Here, the Office Action asserts that MRSA bacteria would be expected to be as vulnerable to visible blue light as any other species of *Gram*-positive bacteria.

However, the Office Action fails to cite any documentary evidence to provide support for this conclusion, either in the cited references or elsewhere. In fact, Kawada *et al.* clearly illustrates this point, indicating that MRSA bacteria are resistant to visible blue light whereas other *Gram*-positive bacteria (*i.e.*, *Propionibacterium acnes*) are not.

The Office Action uses Official Notice to allege this fact in order to make its case for obviousness. While Examiners may rely on Official Notice of facts in lieu of providing documentary evidence in some circumstances, the facts must be "capable of such instant and unquestionable demonstration as to defy dispute" (*In re Ahlert*, 424 F.2d 1088, 1091, 165 U.S.P.Q. 418, 420 (C.C.P.A. 1970), citing *In re Knapp Monarch Co.*, 296 F.2d 230, 132 U.S.P.Q. 6 (C.C.P.A. 1961)). Applicants respectfully submit that the Office Action's conclusion that MRSA bacteria are similar to other *Gram*-positive bacteria and, therefore, would be as susceptible to visible blue light, is *not* "capable of such instant and unquestionable demonstration as to defy dispute." *Id.* Indeed, the teachings of Kawada *et al.* dispute the Office Action's conclusion. Therefore, the Office Action cannot properly rely on such an unsupported allegation of facts in rejecting the claims of the instant application for obviousness.

In summary, a case for obviousness cannot be made in view of the cited prior art for at least the reasons set forth above. Accordingly, Applicants respectfully request that this rejection of the claims be withdrawn.

CONCLUSIONS

In view of the foregoing remarks, the Examiner is respectfully requested to withdraw the rejections of the claims under 35 U.S.C. § 103. Pursuant to 37 C.F.R. § 1.116 and MPEP § 714.12, any amendment that will place the application in condition for allowance may be entered after final rejection. Accordingly, in view of the above remarks, Applicants believe that this application is now ready for allowance. Early notice to this effect is solicited. If in the

opinion of the Examiner a telephone conference would expedite the prosecution of the subject application, the Examiner is invited to call the undersigned.

It is not believed that extensions of time or fees for net addition of claims are required, beyond those that may otherwise be provided for in documents accompanying this paper. However, in the event that additional extensions of time are necessary to allow consideration of this paper, such extensions are hereby petitioned under 37 CFR § 1.136(a), and any fee required therefor (including fees for net addition of claims) is hereby authorized to be charged to Deposit Account No. 16-0605.

Respectfully submitted,

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APPENDIX

Copy of Kawada *et al.* (*Journal of Dermatological Science* (2002) Vol. 30, pp. 129-135)

Submitted concurrently with Amendment After Final filed June 30, 2014, for:

Appl. No.: 11/997,227
Applicant(s): Anderson *et al.*
Filed: July 3, 2008
Confirmation No.: 4281
Examiner: Christopher VanDeusen
Title: INACTIVATION OF GRAM-POSITIVE BACTERIA

Electronic Acknowledgement Receipt

EFS ID:	19447607
Application Number:	11997227
International Application Number:	
Confirmation Number:	4281
Title of Invention:	Inactivation of Gram-Positive Bacteria
First Named Inventor/Applicant Name:	John Galloway Anderson
Customer Number:	826
Filer:	Jeffrey Allen Sunman/Donna Miles
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Attorney Docket Number:	031749/340085
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File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1		340085_Amendment_After_Final.pdf	968369 67ec688af725ca31a9e978f0d094512653d56967	yes	18

	Multipart Description/PDF files in .zip description		
	Document Description	**Start**	**End**
	Amendment Copying Claims - Not in Response to Examiner Suggesting Claims	1	1
	Claims	2	2
	Applicant Arguments/Remarks Made in an Amendment	3	18

Warnings:

Information:

Total Files Size (in bytes):	968369

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

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PATENT APPLICATION FEE DETERMINATION RECORD Substitute for Form PTO-875	Application or Docket Number 11/997,227	Filing Date 07/03/2008	☐ To be Mailed

ENTITY: ☐ LARGE ☒ SMALL ☐ MICRO

APPLICATION AS FILED – PART I

FOR	NUMBER FILED (Column 1)	NUMBER EXTRA (Column 2)	RATE ($)	FEE ($)
☐ BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A	
☐ SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A	N/A	
☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	minus 20 =	*	X $ =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	minus 3 =	*	X $ =	
☐ APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $310 ($155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).			
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))				
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL	

APPLICATION AS AMENDED – PART II

AMENDMENT 06/30/2014	CLAIMS REMAINING AFTER AMENDMENT (Column 1)		HIGHEST NUMBER PREVIOUSLY PAID FOR (Column 2)	PRESENT EXTRA (Column 3)	RATE ($)	ADDITIONAL FEE ($)
Total (37 CFR 1.16(i))	* 2	Minus	** 20	= 0	x $40 =	0
Independent (37 CFR 1.16(h))	* 1	Minus	***3	= 0	x $210 =	0
☐ Application Size Fee (37 CFR 1.16(s))						
☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))						
					TOTAL ADD'L FEE	**0**

AMENDMENT	CLAIMS REMAINING AFTER AMENDMENT (Column 1)		HIGHEST NUMBER PREVIOUSLY PAID FOR (Column 2)	PRESENT EXTRA (Column 3)	RATE ($)	ADDITIONAL FEE ($)
Total (37 CFR 1.16(i))	*	Minus	**	=	X $ =	
Independent (37 CFR 1.16(h))	*	Minus	***	=	X $ =	
☐ Application Size Fee (37 CFR 1.16(s))						
☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))						
					TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.
** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".
*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".
The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.

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/SHAREILL COLES/

This collection of information is required by 37 CFR 1.16. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
11/997,227	07/03/2008	John Galloway Anderson	031749/340085	4281

826 7590 07/23/2014

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ART UNIT	PAPER NUMBER
1774	

NOTIFICATION DATE	DELIVERY MODE
07/23/2014	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

usptomail@alston.com

PTOL-90A (Rev. 04/07)

Advisory Action Before the Filing of an Appeal Brief	Application No. *11/997,227*	Applicant(s) ANDERSON ET AL.	
	Examiner Christopher K. VanDeusen	Art Unit 1774	AIA (First Inventor to File) Status No

--The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

THE REPLY FILED 30 June 2014 FAILS TO PLACE THIS APPLICATION IN CONDITION FOR ALLOWANCE.

NO NOTICE OF APPEAL FILED

1. ☒ The reply was filed after a final rejection. No Notice of Appeal has been filed. To avoid abandonment of this application, applicant must timely file one of the following replies: (1) an amendment, affidavit, or other evidence, which places the application in condition for allowance; (2) a Notice of Appeal (with appeal fee) in compliance with 37 CFR 41.31; or (3) a Request for Continued Examination (RCE) in compliance with 37 CFR 1.114 if this is a utility or plant application. Note that RCEs are not permitted in design applications. The reply must be filed within one of the following time periods:
   a) ☒ The period for reply expires 3 months from the mailing date of the final rejection.
   b) ☐ The period for reply expires on: (1) the mailing date of this Advisory Action; or (2) the date set forth in the final rejection, whichever is later. In no event, however, will the statutory period for reply expire later than SIX MONTHS from the mailing date of the final rejection.
   c) ☐ A prior Advisory Action was mailed more than 3 months after the mailing date of the final rejection in response to a first after-final reply filed within 2 months of the mailing date of the final rejection. The current period for reply expires months from the mailing date of *the prior Advisory Action* or SIX MONTHS from the mailing date of the final rejection, whichever is earlier.
   Examiner Note: If box 1 is checked, check either box (a), (b) or (c). ONLY CHECK BOX (b) WHEN THIS ADVISORY ACTION IS THE FIRST RESPONSE TO APPLICANT'S FIRST AFTER-FINAL REPLY WHICH WAS FILED WITHIN TWO MONTHS OF THE FINAL REJECTION. ONLY CHECK BOX (c) IN THE LIMITED SITUATION SET FORTH UNDER BOX (c). See MPEP 706.07(f).

Extensions of time may be obtained under 37 CFR 1.136(a). The date on which the petition under 37 CFR 1.136(a) and the appropriate extension fee have been filed is the date for purposes of determining the period of extension and the corresponding amount of the fee. The appropriate extension fee under 37 CFR 1.17(a) is calculated from: (1) the expiration date of the shortened statutory period for reply originally set in the final Office action; or (2) as set forth in (b) or (c) above, if checked. Any reply received by the Office later than three months after the mailing date of the final rejection, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

NOTICE OF APPEAL

2. ☐ The Notice of Appeal was filed on _____. A brief in compliance with 37 CFR 41.37 must be filed within two months of the date of filing the Notice of Appeal (37 CFR 41.37(a)), or any extension thereof (37 CFR 41.37(e)), to avoid dismissal of the appeal. Since a Notice of Appeal has been filed, any reply must be filed within the time period set forth in 37 CFR 41.37(a).

AMENDMENTS

3. ☐ The proposed amendments filed after a final rejection, but prior to the date of filing a brief, will not be entered because
   a) ☐ They raise new issues that would require further consideration and/or search (see NOTE below);
   b) ☐ They raise the issue of new matter (see NOTE below);
   c) ☐ They are not deemed to place the application in better form for appeal by materially reducing or simplifying the issues for appeal; and/or
   d) ☐ They present additional claims without canceling a corresponding number of finally rejected claims.
   NOTE: _____. (See 37 CFR 1.116 and 41.33(a)).
4. ☐ The amendments are not in compliance with 37 CFR 1.121. See attached Notice of Non-Compliant Amendment (PTOL-324).
5. ☐ Applicant's reply has overcome the following rejection(s): _____.
6. ☐ Newly proposed or amended claim(s) _____ would be allowable if submitted in a separate, timely filed amendment canceling the non-allowable claim(s).
7. ☒ For purposes of appeal, the proposed amendment(s): (a) ☐ will not be entered, or (b) ☒ will be entered, and an explanation of how the new or amended claims would be rejected is provided below or appended.

AFFIDAVIT OR OTHER EVIDENCE

8. ☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.
9. ☐ The affidavit or other evidence filed after final action, but before or on the date of filing a Notice of Appeal will not be entered because applicant failed to provide a showing of good and sufficient reasons why the affidavit or other evidence is necessary and was not earlier presented. See 37 CFR 1.116(e).
10. ☐ The affidavit or other evidence filed after the date of filing the Notice of Appeal, but prior to the date of filing a brief, will not be entered because the affidavit or other evidence failed to overcome all rejections under appeal and/or appellant fails to provide a showing of good and sufficient reasons why it is necessary and was not earlier presented. See 37 CFR 41.33(d)(1).
11. ☐ The affidavit or other evidence is entered. An explanation of the status of the claims after entry is below or attached.

REQUEST FOR RECONSIDERATION/OTHER

12. ☒ The request for reconsideration has been considered but does NOT place the application in condition for allowance because: See Continuation Sheet.
13. ☐ Note the attached Information *Disclosure Statement*(s). (PTO/SB/08) Paper No(s). _____
14. ☐ Other: _____.

STATUS OF CLAIMS

15. The status of the claim(s) is (or will be) as follows:
   Claim(s) allowed: .
   Claim(s) objected to: .
   Claim(s) rejected: 1 and 6.
   Claim(s) withdrawn from consideration: .

/Christopher K. VanDeusen/
Primary Examiner, Art Unit 1774

Continuation of 11. does NOT place the application in condition for allowance because: Applicant's arguments filed 06/30/2014 have been fully considered but are not persuasive.

Applicant argues that the teachings of the Kawada reference evidences that there was no expectation of success that the claimed methods would have been effective against MRSA, as the results would not have been predictable. The teachings of the Kawada reference have been fully considered but are not persuasive. The Kawada reference makes teachings with respect to a wavelength range of 407-420nm, which is part of the claimed range; however, wavelengths in the range of 400-406nm remain at issue in both of the pending claims. As Kawada does not address the efficacy of these wavelengths, the cited prior art is considered to have a reasonable expectation of success for at least these wavelengths.
With respect to the patient coinfected with MRSA whose condition was worsened by the treatment, Kawada does not attribute this worsening to the patient's infection with MRSA. Lacking a clear causal relationship between MRSA and the worsening of the condition, this teaching by Kawada fails to provide a suitable ground for drawing such a conclusion, and for this reason the argument is not convincing.
With respect to the patients who discontinued the study due to ineffectiveness of the treatment, Kawada merely supposes that the ineffectiveness may have been due to MRSA bacteria which did not respond to blue light. Kawada does not provide any evidenciary support for applicant's claim that MRSA bacteria were not inactivated by the blue light therapy, and as such, this argument is lacking and is not found to be convincing.

Applicant further argues that the cited prior art does not teach that the method of Jones is effective against CNS bacteria. Applicant's argument is supported by the teaching by Kawada that S. epidermis was not reduced by the application of blue light. However, as noted above, the teachings of Kawada are only directed to the application of blue light at wavelengths in the range of 407-420nm; wavelengths in the range of 400-406nm remain at issue in both of the pending claims. As Kawada does not evidence that blue light in the range of 400-406nm is ineffective in treating S. epidermis, the cited prior art remains considered to have a reasonable expectation of success in at least that range of wavelengths.

Applicant further argues that the Office Action has misconstrued the teachings of Jones; specifically, applicant asserts that Jones does not establish the viability of the blue light treatment for bacteria other than P. acnes. This is not convincing; while Jones does describe the use of the blue light therpay in addressing P. acnes, it does not disparage its use for treating other bacteria which contribute to Acne Vulgaris. Further, Jones is not cited as anticipating the claimed limiations, but rather as contributing to the conclusion that the claimed method would have been obvious. As such, while Jones does not teach that S. epidermis is effectively treated by the blue light therapy, its teaching that the therapy can be used to treat, "and at least partially disable or destroy, bacteria that contribute to the cymptons of Acne Vulgaris" is considered to fairly establish that the application of the blue light therapy to such bacteria other than P. acnes would at least be obvious to try and that, failing a teaching to the contrary, that there would be a reasonable expectation of success in such appilcations.

Applicant additionally argues that the Office has misapplied Official Notice. This is not convincing because the Office has not relied upon Official Notice as part of the rejection. The statement that "the same bacterial vulneravility would be exploited in applying the method to MRSA bacteria" is made to clarify the expected outcome of the combined method.

As such, while applicant's arguments have been fully considered, they are not convincing to overcome the previously-cited grounds of rejection of claims 1 and 6, and the claims remain rejected as previously cited.

OK TO ENTER: /C.V./ 07/14/2014

PATENT

RESPONSE UNDER 37 C.F.R. 1.116 - EXPEDITED PROCEDURE - EXAMINING GROUP 1774

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Appl. No.: 11/997,227 Confirmation No.: 4281
Applicant(s): Anderson *et al.*
Filed: July 3, 2008
Art Unit: 1774
Examiner: Christopher VanDeusen
Title: INACTIVATION OF GRAM-POSITIVE BACTERIA

Docket No.: 031749/340085
Customer No.: 00826

Mail Stop AF
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

AMENDMENT AFTER FINAL UNDER 37 C.F.R. § 1.116

Commissioner:

In response to the Final Office Action dated April 25, 2014, please amend the above-identified application as follows:

Amendments to the Claims are reflected in the listing of claims beginning on page 2 of this paper.

Remarks/Arguments begin on page 3 of this paper.

An **Appendix** containing a reference by Kawada *et al.* (*Journal of Dermatological Science* (2002) Vol. 30, pp. 129-135) is attached immediate following page 11 of this paper.

Attorney's Docket No. 031749/340085 PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re: Anderson *et al.* Confirmation No.: 4281
Appl. No.: 11/997,227 Group Art Unit: 1774
Filed: July 3, 2008 Examiner: Christopher VanDeusen
For: INACTIVATION OF GRAM-POSITIVE BACTERIA

Mail Stop RCE
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

INFORMATION DISCLOSURE STATEMENT
UNDER 37 C.F.R. § 1.97(d)

Commissioner:

Attached is a list of documents on form PTO-1449 along with a copy of any cited non-patent literature documents in accordance with 37 C.F.R. § 1.98(a)(2).

It is requested that the Examiner consider these documents and officially make them of record in accordance with the provisions of 37 C.F.R. § 1.97 and Section 609 of the MPEP. By identifying the listed documents, Applicants in no way make any admission as to the prior art status of the listed documents, but are instead identifying the listed documents for the sake of full disclosure.

Respectfully submitted,

/jeffrey a. sunman/

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Substitute for form 1449/PTO (Revised 07/2007)				Complete if Known	
INFORMATION DISCLOSURE STATEMENT BY APPLICANT				Application Number	11/997,227
(Use as many sheets as necessary)				Filing Date	July 3, 2008
				First Named Inventor	John Galloway Anderson
				Art Unit	1774
				Examiner Name	Christopher VanDeusen
Sheet	1	of	2	Attorney Docket Number	031749/340085

U. S. PATENT DOCUMENTS

Examiner Initials*	Cite No.	Document Number Number - Kind Code (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages of Relevant Figures Appear

FOREIGN PATENT DOCUMENTS

Examiner Initials*	Cite No.	Foreign Patent Document Country Code - Number Kind Code (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear	English Language Translation Attached

OTHER DOCUMENTS

Examiner Initials*	Cite No.	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s) , volume-issue number(s), publisher, city and/or country where published.	English Language Translation Attached
	7	POCHI, P. E., "Acne: Androgens and microbiology," *Drug Dev. Res.*, 1988, Vol. 13, pp. 157-168.	
	8	BURKHART, C. G., *et al.*, "Acne: a review of immunologic and microbiologic factors," *Postgraduate Medical Journal*, 1999, Vol. 75, pp. 328-331.	
	9	JAPPE, U., "Pathological mechanisms of acne with special emphasis on *Propionibacterium acnes* and related therapy," *Acta Dermato-Venereologica*, 2003, Vol. 83, pp. 241-248.	
	10	BURKHART, C. N. and GOTTWALD, L., "Assessment of etiologic agents in acne pathogenesis," *Skinmed*, 2003, Vol. 2, No. 4, pp. 222-228.	
	11	TONG, Y., *et al.*, "Population study of atmospheric bacteria at the Fengtai district of Beijing on two representative days," *Aerobiologia,* 1993, Vol. 9, pp. 69-74.	
	12	TONG, Y. and LIGHTHART, B., "Solar radiation is shown to select for pigmented bacteria in the ambient outdoor atmosphere," *Photochemistry and Photobiology,* 1997, Vol. 65, No. 1, pp. 103-106.	
	13	MARSHALL, J. H. and WILMOTH, G. J., "Pigments of *Staphylococcus aureus*, a series of triterpenoid carotenoids," *J. Bacteriology*, 1981, Vol. 147, No. 3, pp. 900-913.	
	14	PELZ, A., *et al.*, "Structure and biosynthesis of staphyloxanthin from *Staphylococcus aureus*," *J. Biol. Chem.*, 2005, Vol. 280, No. 37, pp. 32493-32498.	

Examiner Signature		Date Considered	

*Examiner: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

Substitute for form 1449/PTO (Revised 07/2007)				Complete if Known	
INFORMATION DISCLOSURE STATEMENT BY APPLICANT				Application Number	11/997,227
(Use as many sheets as necessary)				Filing Date	July 3, 2008
				First Named Inventor	John Galloway Anderson
				Art Unit	1774
				Examiner Name	Christopher VanDeusen
Sheet	2	of	2	Attorney Docket Number	031749/340085

OTHER DOCUMENTS

Examiner Initials*	Cite No.	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	English Language Translation Attached
	15	SAKAI, K., *et al.*, "Search for inhibitors of staphyloxanthin production by methicillin-resistant *Staphylcoccus aureus*," *Biol. Pharm. Bull.*, 2012, Vol. 35, No. 1, pp. 48-53.	
	16	CLAUDITZ, A., *et al.*, "Staphyloxanthin plays a role in the fitness of *Staphylococcus aureus* and its ability to cope with oxidative stress," *Infection and Immunity*, 2006, Vol. 74, No. 8, pp. 4950-4953.	

Examiner Signature		Date Considered	

*Examiner: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Appl. No.: 11/997,227 Confirmation No.: 4281
Applicant(s): Anderson *et al.*
Filed: July 3, 2008
Art Unit: 1774
Examiner: Christopher VanDeusen
Title: INACTIVATION OF GRAM-POSITIVE BACTERIA

Docket No.: 031749/340085
Customer No.: 00826

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AMENDMENT UNDER 37 C.F.R. § 1.114

Commissioner:

Responsive to the Advisory Action mailed July 23, 2014, and supplemental to Applicants' Amendment After Final filed June 30, 2014, in response to the Final Office Action mailed April 25, 2014, Applicants respectfully request reconsideration of the claims in view of the following remarks and the Request for Continued Examination pursuant to 37 C.F.R. § 1.114 filed concurrently herewith.

A Complete Listing of the Claims begins on page 2 of this paper.

Remarks/Arguments begin on page 3 of this paper.

An **Appendix** containing a Rule 37 C.F.R. § 1.132 Declaration by Dr. John Anderson, Ph.D. and Exhibits is attached immediately following page 9 of this paper.

Complete Listing of the Claims:

No amendments are currently made to the claims. A complete listing of the pending claims is provided below for the Examiner's convenience and to assist with the Applicants' request for reconsideration of the pending claims.

1. (Previously Presented) A method for disinfecting air, contact surfaces, or materials by inactivating methicillin-resistant *Staphylococcus aureus* (MRSA) in the air, on the contact surfaces, or on the materials, said method comprising exposing the MRSA to visible light without using a photosensitiser, wherein the visible light for inactivating the MRSA consists of wavelengths in the range of 400-420 nm, and wherein the method is performed outside of a human body, the contact surfaces or the materials are non-living, and the air, contact surfaces or materials are not exposed to a disinfecting dose of light at a wavelength above 500 nm.

Claims 2-5. (Canceled)

6. (Previously Presented) A method as claimed in claim 1 wherein the light consists of wavelength 405 nm.

Claims 7-18. (Canceled)

REMARKS/ARGUMENTS

Status of the Claims

Claims 1 and 6 are pending in the application. Reconsideration of these claims is respectfully requested in view of the following remarks and the Request for Continued Examination pursuant to 37 C.F.R § 1.114 filed concurrently herewith. The Examiner's rejections in the Final Office Action mailed April 25, 2014, and the Advisory Action mailed July 23, 2014, are addressed below in the order set forth therein.

The Rejections of the Claims Under 35 U.S.C. § 103 Should Be Withdrawn

Response to Final Office Action

Claims 1 and 6 were rejected under 35 U.S.C. § 103 as being unpatentable over Jones *et al.* (U.S. Application Publication No. 2005/0055070) in view of Burnie *et al.* (U.S. Patent No. 6,627,730) as evidenced by Bek-Thomsen *et al.* (*Journal of Clinical Microbiology* (2008) Vol. 46, No. 10, pages 3355-3360). This rejection of the claims is respectfully traversed for the reasons previously made of record and those further set forth below.

The Office Action draws a number of conclusions from the prior art in order to establish a case for *prima facie* obviousness. However, a person of ordinary skill in the art at the time of the invention would not have reached these conclusions and, consequently, the claimed methods would not have been obvious. In support of this position, Applicants submit concurrently herewith a Rule 37 C.F.R. § 1.132 Declaration by Dr. John Anderson, Ph.D. (hereinafter the "Anderson Declaration"). Dr. Anderson is an inventor of the present application and a person of ordinary skill in the relevant art.

First, the Office Action cites paragraph [0037] of Jones *et al.* as evidence that its method could be used to treat any bacteria that contribute to the symptoms of *Acne Vulgaris* (see, Office Action at page 4). Applicants disagree. In addition to the reasons of record, the Anderson Declaration details why a skilled person would not have read paragraph [0037] of Jones *et al.*, or any other passage therein, and concluded that the method could be used to disinfect bacteria

other than *Propionibacterium acnes* (see, Anderson Declaration paragraphs 4-12). The Anderson Declaration cites a number of references that summarize the state of the art at the time of the invention (see, Exhibits 1-4, also provided as citations 7-10 in the concurrently filed IDS). The consensus of expert scientific and medical opinion at the time of filing was that *P. acnes* was the sole organism responsible for the symptoms of *Acne Vulgaris*. This is consistent with the fact that Jones *et al.* only describes *P. acnes* (and no other bacteria) as contributing to the symptoms of *Acne Vulgaris* (see, for example, paragraphs [0004], [0005], [0023]-[0025], [0029], and [0093]). Moreover, a role for *Staphylococci* sp. in *Acne Vulgaris* had been excluded in the art or was considered negligible.

Therefore, a person of ordinary skill at the time would have concluded that the method of Jones *et al.* was only useful for the treatment of *P. acnes* bacteria in the skin. A skilled person would also have concluded that paragraph [0037] only referred to the treatment of *P. acnes* and certainly not *S. epidermis* as proposed by the Office Action. Accordingly, the Office Action's conclusion is flawed.

The Office Action also asserts that Bek-Thomsen provides evidence that *Staphylococcus epidermis* bacteria contribute to the symptoms of *Acne Vulgaris* and, therefore, could be disinfected by the method of Jones *et al.* (see, Office Action at page 4). Applicants disagree.

Even if paragraph [0037] of Jones *et al.* encompassed bacteria other than *P. acnes* (which Applicants assert it does not), the Anderson Declaration describes why a person of ordinary skill in the art would not have concluded from Bek-Thomsen that *S. epidermis* bacteria contribute to the symptoms of *Acne Vulgaris* (see, Anderson Declaration paragraphs 13-15). Rather, Bek-Thomsen only "raises the question of the potential role of this species" in *Acne Vulgaris* and states that "a reevaluation of the role of *S. epidermis* may be advisable." In fact, in the same paragraph, Bek-Thomsen explicitly acknowledges that the prior art had excluded *Staphylococci* as agents that play a role in the pathogenesis of *Acne Vulgaris* (see, page 3359, left column, paragraph 4).

Therefore, given the teachings of Bek-Thomson, and the state of the art at the time of the invention, a person of ordinary skill would not have concluded from this reference that *S. epidermis* contributes to the symptoms of *Acne Vulgaris*, as alleged by the Office Action.

Consequently, even if paragraph [0037] of Jones *et al.* did encompass the treatment of bacteria other than *P. acnes* (which Applicants assert it does not), *S. epidermis* would not have been considered a bacteria that contributes to the symptoms of *Acne Vulgaris*. Accordingly, the Office Action's conclusion is flawed.

The Office Action further asserts that *S. epidermis* and MRSA are similar and would be expected to have the same vulnerability to visible blue light of the claimed wavelengths (see, Office Action at pages 5-7). Applicants disagree.

The Anderson Declaration asserts that a person of ordinary skill would not have expected *Staphylococcus* sp., including MRSA, to be vulnerable to the visible blue light used by the method of Jones *et al.* (see, Anderson Declaration paragraphs 16-22). The Anderson Declaration cites a number of references in support of this position that summarize the state of the art at the time of the invention (see, Exhibits 5-10, also provided as citations 11-16 in the concurrently filed IDS). Given the state of the art, a skilled person would have had no expectation that the method described by Jones *et al.*, which is designed for use against the oxygen-sensitive, anaerobic bacterium *P. acnes*, would have been effective against *Staphylococcus* sp., including MRSA. Accordingly, the Office Action's conclusion is flawed.

In summary, a person of ordinary skill in the art at the time of the invention would not have reached any of the conclusions described above, which were relied upon by the Office Action to establish a case for *prima facie* obviousness. A skilled person would not have concluded **(i)** that the method of Jones *et al.* could have been used to treat bacteria other than *P. acnes*, **(ii)** that *S. epidermis* bacteria contribute to the symptoms of *Acne Vulgaris* and could have been disinfected by the method of Jones *et al.*, or **(iii)** that *S. epidermis* and MRSA are similar enough that one would expect the method of Jones *et al.* to be effective against MRSA. For these reasons, and those previously made of record, Applicants respectfully submit that this rejection under 35 U.S.C. § 103(a) for obviousness should be withdrawn.

Response to Advisory Action

The Advisory Action maintained the rejection for obviousness set forth in the Final Office Action. Applicants disagree with the reasons stated at page 2 of the Advisory Action.

In the Amendment filed June 30, 2014, Applicants submitted that disinfection of MRSA bacteria using visible blue light would not have been predictable and that there would have been no expectation of success. In support of this position, Applicants submitted a prior art reference by Kawada *et al.* (*Journal of Dermatological Science* (2002) Vol. 30, pp. 129-135).

Kawada *et al.* was cited as teaching (**i**) that visible blue light was not effective in treating a patient that was co-infected with MRSA bacteria (see, page 133), (**ii**) that MRSA bacteria were not inactivated by the use of visible blue light (see, page 134), and (**iii**) that visible blue light treatment was effective against *P. acnes* but had no effect on *S. epidermis* bacteria (see, Table 3 and page 133).

The Advisory Action states that a wavelength range of 407-420 nm is taught by Kawada *et al.*; however, wavelengths in the range of 400-406 nm are allegedly not addressed by the reference. Therefore, the Advisory Action asserts that wavelengths in the range of 400-406 nm remain at issue in the present claims and that the prior art is considered to have a reasonable expectation of success for the disinfection of *S. epidermis* and the disinfection of MRSA at wavelengths between 400-406 nm. Applicants disagree.

In section 2.2, Kawada *et al.* indicates that the light source employed was a ClearLight™ (Lumenis, Tokyo) (see, page 133). The reference states that this light source had double UV-cut filters and an emitting peak of 407-420 nm. However, it is clear from the emission spectrum shown in Figure 1 that the light source has emitting peaks <u>other</u> than 407-420 nm. Specifically, Figure 1 illustrates that the UV-cut filters used by Kawada *et al.* blocked emissions <u>below 400 nm</u> (*i.e.*, UV light) but allowed emissions of wavelengths ranging from <u>400 nm to at least 800 nm</u>. Thus, the light source used in each experiment by Kawada *et al.* also included emissions in the range of <u>400-406 nm</u>.

As such, the cultured *P. acnes* and *S. epidermis* bacteria described in Table 3 of Kawada *et al.* were treated with visible light at wavelengths between <u>400-420 nm</u>. This range of wavelengths was effective against *P. acnes* but had <u>no effect on *S. epidermis*</u>. Therefore, it would have been known in the art at the time of filing that the method of Jones *et al.* <u>would not have been effective against *S. epidermis*</u>.

Thus, the Office Action's case for obviousness cannot be made, as it relies on the conclusion that the method of Jones *et al.* would have been effective against *S. epidermis* bacteria. On the contrary, Kawada *et al.* evidences that a person of ordinary skill in the art would have had no motivation to treat *S. epidermis* bacteria with 400-420 nm light because there would have been no reasonable expectation of success.

Similarly, Kawada *et al.* also teaches that patient skin, co-infected with MRSA, was treated with visible light at wavelengths between 400-420 nm. As previously discussed, Kawada *et al.* states that MRSA- and MSSA-infected patients discontinued treatment due to "worsened" symptoms (see, page 133) and the "ineffectiveness of phototherapy" (see, page 134). Kawada *et al.* further states that "[t]he main pathogen in acne lesions of these patients may have been *S. aureus* that did not respond to blue light" (see, page 134).

Therefore, the reference suggests that visible light in the range of 400-420 nm was ineffective against MRSA bacteria in the skin. As such, Applicants' claimed method would not have been predictable based on the state of the art at the time of filing, as there would have been no expectation of success that 400-420 nm visible light could disinfect MRSA bacteria.

The Advisory Action also asserts that Kawada *et al.* lacks a clear causal relationship between MRSA infection and the worsening of the patient's condition. The Advisory Action states that Kawada *et al.* "merely supposes" that the ineffectiveness of treatment may have been due to MRSA bacteria which did not respond to blue light without providing an evidentiary support for this claim. For this reason, Applicants' argument was found unpersuasive.

However, Kawada *et al.* explicitly teaches that patients infected with MRSA or MSSA had to discontinue therapy due to worsened symptoms. Kawada *et al.* then clearly suggests that the *S. aureus* bacteria infecting these lesions (*i.e.*, MRSA or MSSA) do not respond to blue light therapy. Although direct evidence is not provided to support this claim, a person of ordinary skill in the art, having considered these teachings of Kawada *et al.*, would have had no reasonable expectation that visible light between 400-420 nm would be effective against MRSA bacteria. Therefore, Applicants' claimed method was unpredictable in view of the art at the time of filing, and there would have been no expectation of success.

The Advisory Action further asserts that the method of Jones *et al.* is not limited to use with *P. acnes* bacteria and, though it does not teach the treatment of *S. epidermis*, paragraph [0037] can be broadly interpreted to include any bacteria that contribute to the symptoms of *Acne Vulgaris*. Applicants disagree with this conclusion for the reasons previously made of record and those further set forth above.

Finally, the Advisory Action asserts that the Office Action did not rely on Official Notice in order to establish the rejection for obviousness. The Advisory Action states that the quoted passage is made "to clarify the expected outcome of the combined method." Applicants respectfully disagree.

Here, in the absence of any documentary evidence in the cited prior art or elsewhere, the Examiner alleges what the "expected" outcome of the method will be in order to establish a reasonable expectation of success. While Examiners may rely on Official Notice of facts in lieu of providing documentary evidence in some circumstances, the facts must be "capable of such instant and unquestionable demonstration as to defy dispute" (*In re Ahlert*, 424 F.2d 1088, 1091, 165 U.S.P.Q. 418, 420 (C.C.P.A. 1970), citing *In re Knapp Monarch Co.*, 296 F.2d 230, 132 U.S.P.Q. 6 (C.C.P.A. 1961)). The Office Action's conclusion that MRSA bacteria are similar to other *Gram*-positive bacteria and, therefore, would be as susceptible to visible blue light, is not "capable of such instant and unquestionable demonstration as to defy dispute." *Id.* This is at least evidenced by the Anderson Declaration, which describes why a person of ordinary skill in the art would not have expected visible light to disinfect *Staphylococci* sp., including MRSA bacteria. Therefore, the Office Action cannot properly rely on such an unsupported allegation of facts in rejecting the claims of the instant application for obviousness.

For the reasons set forth above, and those previously made of record, Applicants submit that a case for *prima facie* obviousness cannot be made. Accordingly, Applicants respectfully request that this rejection of the claims under 35 U.S.C. § 103(a) be withdrawn.

CONCLUSIONS

In view of the foregoing remarks, the Examiner is respectfully requested to withdraw the rejections of the claims under 35 U.S.C. § 103. Applicants believe that this application is now ready for allowance. Early notice to this effect is solicited. If in the opinion of the Examiner a telephone conference would expedite the prosecution of the subject application, the Examiner is invited to call the undersigned.

It is not believed that extensions of time or fees for net addition of claims are required, beyond those that may otherwise be provided for in documents accompanying this paper. However, in the event that additional extensions of time are necessary to allow consideration of this paper, such extensions are hereby petitioned under 37 C.F.R. § 1.136(a), and any fee required therefor (including fees for net addition of claims) is hereby authorized to be charged to Deposit Account No. 16-0605.

Respectfully submitted,

/jeffrey a. sunman/

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LEGAL02/34814107v1

<u>APPENDIX</u>

Rule 37 C.F.R. § 1.132 Declaration by Dr. John Anderson, Ph.D. and Exhibits

Appl. No.:	11/997,227	Confirmation No.: 4281
Applicant(s):	Anderson *et al.*	
Filed:	July 3, 2008	
Art Unit:	1774	
Examiner:	Christopher VanDeusen	
Title:	INACTIVATION OF GRAM-POSITIVE BACTERIA	

Attorney's Docket No. 031749/340085 PATENTS

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re:	Anderson et al.	Confirmation No.:	4281
Appl. No.:	11/997,227	Group Art Unit:	1774
Filed:	July 3, 2008	Examiner:	Christopher Vandeusen
For:	INACTIVATION OF GRAM-POSITIVE BACTERIA		

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Commissioner for Patents
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Alexandria, VA 22313-1450

RULE 37 C.F.R. § 1.132 DECLARATION OF JOHN ANDERSON

I, John Anderson, do hereby declare and say as follows:

1. I, John Anderson, received the B.Sc. and Ph.D. degrees from the University of Strathclyde, Glasgow, U.K., in 1968 and 1971, respectively. I was appointed a Lecturer in Microbiology in 1971. In 1979, I became a Senior Lecturer, with a promotion to Reader and Professor of Microbiology, in 1992 and 2002, respectively. I was Head of the Department of Bioscience at Strathclyde University from 2003-2007. I remained at the University of Strathclyde where I am currently an Emeritus Professor of the Strathclyde Institute of Pharmacy and Biomedical Sciences and a Research Associate in the Department of Electronic and Electrical Engineering as well as being Co-director of The Robertson Trust Laboratory for Electronic Sterilisation Technologies (ROLEST). My research interests include biomedical, environmental, and food microbiology, and most recently interdisciplinary microbiological and electrical engineering research on electronic sterilisation technologies with over 200 journal and conference papers published in these and associated areas.

2. I have read and understand the specification of the captioned application and the pending claims in the application.

3. In the Final Office Action and the recently issued Advisory Action, the Examiner appears to be making three points. These can be summarized as follows:

(i) Jones teaches a method that can be used to treat any bacteria that contribute to the symptoms of *Acne Vulgaris*;

(ii) from Bek-Thomsen, one of skill understands that *S. epidermidis* contributes to Acne Vulgaris; and

(iii) *S. epidermidis* and MRSA are similar enough that one would expect them to have the same vulnerability to visible blue light of the claimed wavelengths.

4. Regarding point (i), I assert that the Office Action has misconstrued the teachings of Jones. In my opinion it is clear that Jones does not establish the viability of a blue light treatment for anything other than *P. acnes* present on the skin.

5. The Examiner points to the text of paragraph [0037] and states that from this it is clear that the method of Jones would be understood as generally applicable to all bacteria, as blue light based therapies can be used to treat "and at least partially disable or destroy, bacteria that contribute to the symptoms of Acne Vulgaris". The assumption made from this statement is that *Staphylococcus* is a bacteria which causes the symptoms of *Acne Vulgaris*.

6. However, based on the state of the art at the time of filing, the skilled person would be firmly of the opinion that *Acne Vulgaris* is caused by *P. acnes*. Indeed, the consensus of expert scientific and medical opinion was (and is) that *P. acnes* is the responsible organism. This causal association goes back many decades and illustrative examples can be located in numerous publications available prior to the filing date of the present application.

7. For example Pochi (1988) (Exhibit 1) describes *P. acnes* as the bacterium responsible for the formation of the inflammatory lesions and that in acne, **only** *P. acnes* is considered to be of pathogenic importance.

8. In a review of immunologic and microbiologic factors involved in acne, Burkhart *et al.* (1999) (Exhibit 2) describe *P. acnes* as the "acne-causing organism" via its effects on humoral and cell-mediated immunity, complement activation and cytotoxin production. Furthermore, Burkhart *et al* (1999) considers the role of *Staphylococci* to be **negligible** and as evidence of this is the fact that antibiotics that selectively eliminated these organisms did not affect the clinical response of patients.

9. Also, in a review of the pathological mechanisms on acne, Jappe (2003) (Exhibit 3) describes the key role of *P. acnes* in acne and **excludes** a role for *Staphylococci*.

10. Additionally, in an assessment of etiological agents in acne pathogenesis, Burkhart and Gottwald (2003) (Exhibit 4) describe that the dermal inflammation associated with acne is not due to the presence of bacteria but from biologically active mediators produced by *P. acnes.*

11. While I would not doubt that numerous *Staphylococcal* species are present as commensals on human skin and that when investigating the pathology of acne, *Staphylococci* may also be isolated, it is clear from the prior art that one of skill would consider *P. acnes* **as the sole and principle agent responsible for the symptoms of Acne Vulgaris**.

12. Together with the fact that Jones is exclusively directed to the inactivation of *P. acnes*, it stands that the statement in Jones reading (in full – see [0037]) "....the apparatus may be used to treat a region of skin affected by the condition Acne Vulgaris by causing a photochemical reaction in said region that stimulates the production of free radicals that react with and at least partially disable or destroy, bacteria that contribute to the symptoms of Acne Vulgaris" would (and should) be interpreted as a mere confirmation that the apparatus may be effective against *P. acnes* – this being **the** (only) organism which contributes to the symptoms of *Acne Vulgaris.*

13. Regarding point (ii), based on the flawed interpretation of Jones, the Examiner introduces the paper by Bek-Thomsen *et al* (2008) as evidence that *Staphylococcus epidermidis* contributes to the symptoms of Acne. In addition to the arguments presented above, it is important to note that not only was this document published some 3 years after the priority date of the present application, it involves a small 5 patient study and did not claim that *S. epidermidis* contributes to the symptoms of acne; rather it raised this as a possibility that required further study.

14. In any case, if the Examiner is in possession of a proper argument, then it would follow that having considered the disclosure of Bek-Thomsen, one of skill would understand from the teaching of Jones, that blue light could be used to inactivate *Staphylococci*. Not least because Bek-Thomsen was not available at the time of filing, I submit that this is not the case, as Jones has nothing to do with *Staphylococci* and thus even if one of skill were to conclude from Bek-Thomsen that *Staphylococcus epidermidis* contributed to Acne Vulgaris (which he cannot), Jones makes no claims as to the effectiveness of blue light against *Staphylococcus* sp.

15. Based on the above, it is clear that the teaching of Bek-Thomsen relied upon by the Examiner is inconsistent with the teaching of the scientific literature available at the time of filing. Moreover I assert that the skilled person would not read Jones and understand that *Staphylococci* were a factor in *Acne Vulgaris*, he would not (and could not) consult Bek-Thomsen, and he would not apply blue light as a means to inactivate *Staphylococcus* sp.

16. Regarding point (iii), even if one were to concede (which I do not) that the Jones reference extends to any bacteria, for the reasons set out below, it would not be at all obvious to the skilled person that visible (blue) light in the claimed range would have any effect on the viability of *Staphylococcus* sp., including MRSA.

17. It is known that many *Staphylococci* contain extractable pigments.. The pigment can be a carotenoid-type pigment which allows pigmented strains of *Staphylococcus* species, including *S. epidermidis* and *S. aureus*, to survive exposure to visible light. It is thought that the carotenoid-type pigments protect them from the damaging effects of solar radiation.

18. It stands to reason that even if the skilled person were to have considered the teaching of Jones and were to have assumed that *Staphylococci* sp. were involved in *Acne Vulgaris* (a point I strongly contest), given the generally pigmented status of the *Staphylococci*, he would have thought them intrinsically resistant to visible light.

19. By way of support, I would refer to (Tong et al, 1993) (Exhibit 5) which discloses that *Staphylococcus* species, including *S. aureus*, have been found to be prominent in atmospheric air and that this is likely due to the presence of carotenoid pigments that protect the bacteria from near UV and visible light damage by quenching triplet-state photosensitizers and reactive oxygen species (see also Tong and Lighthart, 1997) (Exhibit 6).

20. The "golden" carotenoid pigment present in *S. aureus*, including MRSA, has been characterised as a triterpenoid carotenoid referred to as staphyloxanthin (Marshall and Wilmoth, 1981; Pelz et al, 2005) (Exhibit 7 and Exhibit 8). More recent work has provided further evidence that Staphyloxanthin, which is produced by MRSA (Sakai et al (2012)) (Exhibit 9), is now considered to be a virulence factor that protects the organism from killing by oxidative stress and reactive oxygen species (Clauditz et al, 2006) (Exhibit 10).

21. This greater tolerance to light and reactive oxygen species associated with *Staphylococcus* species is in stark contrast to the effects of light on *P. acnes*. Unlike *S. aureus*, *P. acnes* is an oxygen sensitive anaerobic bacterium, and, as described by Jones in paragraphs [0023] and [0024], it is easily harmed or damaged by the presence of oxygen and is destroyed by a photochemical reaction that results in the production of free radicals such as singlet oxygen (a reactive oxygen species).

22. Based on knowledge of the pigmented status alone, one skilled in the art would not believe that the methods described by Jones *et al.*, which were specifically designed to inactivate *P. acnes*, could be applied to *Staphylococcus* species including MRSA.

23. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

24th September 2014

John Anderson

Date

EXHIBIT 1

Drug Development Research 13:157-168 (1988)

Acne: Androgens and Microbiology

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ABSTRACT

Pochi, P.E.: Acne: Androgens and microbiology. Drug Dev. Res. **13**:157–168, 1988.

Acne, the most prevalent disease of the skin, is a multifactorial follicular disorder in which androgens and microbiology are essential for the occurrence of the pathogenic events that characterize it. The role of the androgen is to stimulate the development and secretory activity of the sebaceous gland, which performs several important actions in the etiopathogenesis of acne. The anaerobic diphtheroid *Propionibacterium acnes,* a resident organism in acne-susceptible follicles, is directly responsible for much of the inflammation in acne. This review deals with a description of these events and the therapeutic modalities that have been developed in an attempt to counteract them.

Key words: antiandrogen, *Propionibacterium acnes,* sebaceous gland

INTRODUCTION

Acne is a highly prevalent skin disorder affecting a large segment of the population, with a peak incidence at age 17 [Pochi, 1985a]. In most individuals, it is a self-limited disease, although its duration may range from a few months to several years. The condition is asymptomatic and anatomically involves the face and upper trunk. Clinically, the lesions that form may be noninflammatory (comedones) or inflammatory (papules, pustules, and nodules or "cysts"). In some patients, the disease is of minimal severity and ordinarily only a minor cosmetic nuisance; in others, it is more highly inflammatory and, thus, not only a significant clinical entity but also one that may lead to emotional problems, or at the very least, some difficulty in social adjustment.

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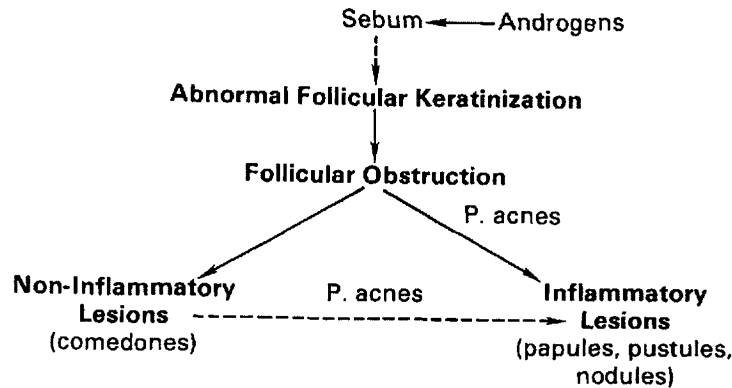

Fig. 1. Sequence of pathologic events in acne, starting from abnormal follicular keratinization, the primary known alteration in the disease, to the development of inflammatory lesions.

PATHOGENESIS

The cause of acne is unknown, although much information has accumulated in recent years that has elucidated, to a considerable extent, the nature of the pathologic events in the disease [Shalita et al., 1987].

Acne affects specialized pilosebaceous units, termed sebaceous follicles, that are located in the skin of the face and trunk. These follicles have wide lumens and large sebaceous glands associated with them, and they are heavily colonized by facultatively anaerobic, gram-positive diphtheroids, known as *Propionibacterium acnes*. As will be discussed below, these bacteria are responsible for the formation of the inflammatory lesions. The sebaceous glands are holocrine structures and secrete large quantities of *sebum* into the follicular lumen and thence to the skin surface. Sebum, found in all mammalian species, is a complex mixture of lipids with a composition unique for man, consisting of, in decreasing order of concentration, triglycerides (60%), wax esters (25%), squalene (12%), and sterols and sterol esters (less than 5%) [Downing et al., 1969; Green et al., 1984]. The purpose of the presence of sebaceous glands in man is not clear and may represent an atavistic happenstance, whereas in animals, sebum is important for coating the fur, heat insulation, territorial marking, and sexual attraction [Kligman, 1963].

The initial detectable alteration in acne is the abnormal retention of scales in the keratinizing follicular epithelium of susceptible pilosebaceous follicles (Fig. 1). Normally, the cells of this desquamating layer are shed readily, but in acne there is a retention hyperkeratosis that leads to retarded shedding of skin scales in large sheets that become entrapped in the follicle lumen [Knutson, 1974]. The result is follicular obstruction and the formation of a *comedo* that at this stage is not visible and is, therefore, termed a *microcomedo*, consisting of aggregated keratin sheets and fragments, microorganisms (primarily *P. acnes*), and sebum. From this point on, several events can occur. The microcomedo may remain relatively unchanged, or it may enlarge to form a visible comedo, either a closed comedo ("whitehead") or a further-stage lesion, the open comedo ("blackhead"). Inflammatory lesions arise largely in microcomedones, occasionally from closed comedones and only rarely from open comedones [Kligman, 1974]. Thus, most inflammatory acne lesions develop in follicles that on surface inspection look normal.

The stimulus for the keratinizing follicular abnormality in acne is not known, although it has been speculated that sebum itself might be responsible [Pochi, 1984a]. The basis for this assertion is the finding that linoleate levels in sebum are low in acne [Morello et al., 1976].

In fact, detailed analyses of the fatty acids of triglycerides and wax esters of sebum have disclosed this to be the only detectable difference between acne and nonacne subjects. The assumption is that as sebum secretion begins to increase in mid-to-late childhood under the influence of androgens, the linoleate concentration in sebum becomes relatively low [Stewart et al., 1986]. This circumstance could then lead to the follicular retention hyperkeratosis, since it is known that excess desquamation of the skin surface occurs in individuals with essential fatty acid deficiency [Downing et al., 1986]. Even if this hypothesis were correct, however, it would not explain: 1) why acne is not a manifestation of essential fatty acid deficiency; or 2) why all individuals do not develop acne. This means that either there is genetic susceptibility, as yet unidentified, for this change to take place or that the other known pathogenic factors in acne are more dominant, e.g., the amount of sebum produced, host-defense mechanisms in response to inflammatory stimuli, etc.

ANDROGENS

Role in Acne

As discussed above, sebum is of probable, or at least putative, importance in the induction of the early abnormal events in the formation of the acne lesion. It has other important actions as well [Downing et al., 1987]. For one, it is the substrate for the growth and proliferation of *P. acnes,* which is dependent upon the triglycerides of sebum for its nutrition [Rebello and Hawk, 1978]. Also, the liberated free fatty acids are cytotoxic agents that probably contribute to the inflammatory reaction [Tucker et al., 1980]. Finally, treatment modalities that can substantially reduce sebaceous gland activity will almost invariably improve acne [Cunliffe, 1987].

Androgens are important in acne for their role in the stimulation of sebaceous gland development [Pochi, 1982a,b]. While it is not absolutely certain that the only effect of androgens in the pathogenesis of acne is its sebum-stimulating property, it is undoubtedly a major one. In man, the sebaceous glands develop embryologically between the 13th and 15th week of fetal life [Serri and Huber, 1963]. At birth, the glands are fairly well developed, full maturation having occurred by the sixth fetal month. The source of androgenic stimulation is probably the fetal endocrine system, which provides sources of androgen production, namely, the fetal adrenal cortex and the gonads. Acne may be present at birth in a diminutive form ("acne neonatorum") but subsides within a few months as sebum secretion and androgen levels decline in early infancy [Pochi, 1982c].

During childhood, sebum levels remain very low but begin to increase as early as age 6 in girls and age 7 in boys [Bloch, 1931; Pochi et al., 1977; Stewart and Downing, 1985]. This event coincides with the elaboration of adrenocortical androgens (adrenarche). This increase in sebum is subtle but nonetheless measurable and occurs prior to significant bacterial colonization of the sebaceous follicle by *P. acnes* [Leyden et al., 1975]. In fact, at this early stage, a few small comedones can be observed in some individuals [Bloch, 1931], thus rendering unlikely the possibility that an increase in *P. acnes* effects faulty keratinization. In support of this are ultrastructural studies that have disclosed the presence of abnormal follicular hyperkeratosis without evidence of bacterial colonization [Lavker et al., 1981].

With elaboration of gonadal androgens in early puberty, sebum secretion continues to increase, reaching a peak at approximately age 17 to 18 [Pochi et al., 1979], a time, as already mentioned, that acne reaches, on average, peak severity [Kraning and Odland, 1979]. Although androgen and sebum levels do not further change to any appreciable degree in the third and fourth decades of life [Pochi and Strauss, 1974], acne will almost always have disappeared by this time. Thus the explanation for the natural involution of acne does not appear to have an endocrine basis.

More direct evidence of the role of androgens in sebum and acne lies in the observation that acne does not occur in castrated men, that sebaceous gland secretion in this group is lower

than normal, and that androgen administration to individuals with weakly stimulated sebaceous glands (children, castrated men, postmenopausal women) increases sebaceous gland activity [Pochi and Strauss, 1974].

In acne, sebum secretion is higher than normal [Cunliffe and Shuster, 1969; Pochi and Strauss, 1974; Harris et al., 1983], but overlap does occur between those with and without acne. Androgen levels, viz. testosterone, free testosterone, and dehydroepiandrosterone sulfate, may also be increased in acne, at least in women [Marynick et al., 1983; Lucky et al., 1983; Held et al., 1984], but no correlation can be established between sebum production levels and testosterone or other androgens [Pochi and Strauss, 1974]. While seemingly contradictory, there are reasons why this might be so. First, the androgenic influence on the sebaceous gland might be modulated by other endocrine substances, e.g., pituitary hormones, thyroid hormones, and cortisol, all of which appear to act synergistically with androgens to permit the sebaceous gland to respond optimally to androgen stimulation [Pochi and Strauss, 1974]. Second, the peripheral metabolism of androgens at the target site may differ from person to person, so that androgen levels may not correlate positively with sebum levels. Sansone and Reisner showed that skin of acne patients had increased 5α-reductase levels when incubated with radioactive testosterone [Sansone and Reisner, 1971]. While it has never been clear whether this is merely a reflection of the larger amount of sebaceous gland tissue apt to be found in the skin of acne patients or the result of enhanced metabolism of androgen, it has been demonstrated that acne patients, like those with hirsutism, have higher circulating levels of 3α-androstanediol glucuronide (3α-diol) [Lookingbill et al., 1985], an intracytoplasmic metabolite of dihydrotestosterone, the 5α-reduced intracellular androgenic metabolite of testosterone [Bruchovsky and Wilson, 1968]. But once again, one could forcibly argue that increased 3α-diol glucuronide levels only indicate the presence of a larger volume of androgen-sensitive sebaceous gland tissue and would not explain why the tissue is overactive in the first place.

Therapeutic Considerations

The well-established link between androgens and acne has, for many years, led researchers to reduce sebaceous gland secretion by treatment modalities that would decrease androgen stimulation of the sebaceous glands.

Estrogen. Systemically administered estrogen has been the treatment longest in use for the treatment of acne. Such treatment is applicable only to women because of the feminizing effects of estrogen in men. The most favored use of estrogen is its cyclic administration in the form of oral contraceptive medication [Strauss and Pochi, 1967]. The mechanism of action is inhibition of luteinizing hormone and ovarian androgen production. The doses required to achieve sebaceous gland inhibition—and inconsistent at that—exceed 50 μg daily of ethinyl estradiol [Pochi and Strauss, 1973]. With the recognition of serious systemic complications with doses of estrogen greater than 50 μg per day (which may occur rarely) [Wessler, 1980], most women receive a 50-μg or lower dose for contraceptive purposes. Few women with acne will have their disease improve from these small dose regimens [Pochi, 1983].

Topically, estrogen has little or no effect on sebum or acne. Although, experimentally, high concentrations can achieve significant sebaceous gland inhibition [Strauss and Pochi, 1963], its effect is not exerted at the local site but via systemic absorption and its action on the pituitary-ovarian axis. Yet studies in animals have shown that estrogen can alter sebaceous gland secretion locally [Ebling and Skinner, 1983]; the possibility remains that estrogen analogues may at some future time be found to be helpful in this regard.

Glucocorticoid. The administration of low daily doses of cortisol derivatives, such as prednisone or dexamethasone, can decrease adrenal androgen levels [Nader et al., 1984]. Of particular interest is the androgen dehydroepiandrosterone sulfate (DHEA-S), which, in one study, was found to be increased in the blood in the majority of patients, men or women, with

TABLE 1. Topical Antiandrogens

Δ'-Chlormadinone acetate
Clometherone
Cyproterone acetate
Flutamide
2-Formyl-prednisolone-21-acetate
11-Hydroxyprogesterone
17α-Methyl-B-nortestosterone
A-norprogesterone
Progesterone
17α-Propylmesterolone
Spironolactone

severe acne [Marynick et al., 1983]. On low-dose adrenocortical suppression, viz. 0.25–0.5 mg of dexamethasone nightly, most patients improved. However, no placebo groups were included, and the possibility of an antiinflammatory effect rather than a hormone effect cannot be discounted. In another study, 0.5 mg of dexamethasone a day decreased DHEA-S blood levels by an average of 80%, but one-third of the patients failed to improve [Pochi, 1984b]. No correlation could be established between decreased DHEA-S levels and decreased acne activity.

Marked suppression of sebum has been observed when estrogen and glucocorticoid have been simultaneously administered [Pochi and Strauss, 1976]. In a small clinical study, complete clearing of severe acne in women was noted from the administration of 50 μg of ethinyl estradiol and 5 mg prednisone daily; sebum decreased by 50% [Saihan and Burton, 1980].

Antiandrogens. Antiandrogens interfere with the action of androgens at their target tissues [Neumann, 1983; Lucky, 1985]. Testosterone in its free (unbound) form enters the cell and is reduced to dihydrotestosterone (DHT), which is then bound to protein receptors. The conversion of testosterone to DHT is catalyzed by the enzyme 5α-reductase. The DHT receptor complex is translocated to the cell nucleus where it binds to chromatin and stimulates RNA synthesis, which in turn regulates the physiologic activity of the target cell. This sequence of events can be inhibited by antiandrogens in one of two principal ways, either by interfering with the reduction of testosterone to DHT or with the binding of DHT to receptor protein.

Cyproterone acetate was one of the first antiandrogens to be introduced into clinical practice for the treatment of hirsutism in women [Hammerstein and Cupceancu, 1969]. It was administered in a "reversed sequential" regimen in a dose of 100 mg per day from days 10 to 25 of the menstrual cycle, together with estrogen 50 μg daily from days 5 to 25. It was later shown to be effective for treating acne as well [Hammerstein et al., 1975], but a lower dose of cyproterone acetate, 2 mg daily for 3 weeks, combined with 35 μg of ethinyl estradiol, a combination product marketed abroad for the treatment of acne, is less effective [Greenwood et al., 1985].

Topically, cyproterone acetate has not proved to be effective in reducing sebum or acne [Cunliffe et al., 1969], despite its ability to inhibit DHT receptor binding and its clearly demonstrated sebum-suppresive effect when administered orally [Ebling and Fanta, 1982]. The same lack of response, either in a substantive reduction of sebum or in clinical improvement of acne in control studies, has been a consistent finding with a variety of steroidal and nonsteroidal antiandrogens (Table 1) [Strauss and Pochi, 1970; Pye et al., 1976; Simpson et al., 1979; Hellgren et al., 1982; Lyons and Shuster, 1982; Tamm et al., 1982; Walton et al., 1986; Schmidt and Spona, 1987].

No unifying explanation is at hand that would clarify the seeming failure of topical

antiandrogens, although they clearly have an antiandrogenic effect when tested in a variety of animal sebaceous gland assays (hamster flank organ, hamster ear glands, and rat cutaneous sebaceous glands, together with a range of specific methodologies used in assays in these sebaceous tissues) [Neumann and Töpert, 1986]. One can speculate on the reasons for the largely negative results to date: lack of absorption down to the germinative cell epithelium of the sebaceous gland or, conversely, too rapid absorption, allowing for insufficient concentration at the specific target locus; inactivation by being bound to enzymes or conjugated to inactive metabolites; and the fact that antiandrogen is not the actual pharmacologically active form but rather an endogenous metabolite formed in the liver or noncutaneous extrahepatic tissues. Future research aimed at discovering effective topical locally active antiandrogens for acne and other androgen-dependent cutaneous disorders, such as hirsutism, androgenetic alopecia, and axillary odor, should address the issues mentioned above.

MICROBIOLOGY

Role in Acne

In the last century, acne was considered a microbial disorder, for the obvious reason that the lesions often contained pus. The histologic observation in 1896 that bacilli were present in the follicles of acne lesions [Unna, 1896] and their subsequent isolation by culture [Sabouraud, 1897] confirmed this suspicion. Then, when bacilli were found in the follicles of individuals without acne, their role in acne was dismissed [Lovejoy and Hastings, 1911].

In the past three decades, however, investigations have made it abundantly clear that *P. acnes* is indeed important in acne [Pochi et al., 1981; Puhvel, 1982]. *P. acnes* occurs in large numbers in the sebaceous follicles of the face and trunk but is sparse and absent elsewhere on the skin [McGinley et al., 1978]. Animals do not have acne, a fact that has considerably hindered the study of the inflammation from this bacterium; moreover, animal seba, low in triglyceride content, cannot support the growth of *P. acnes* [Webster et al., 1981]. However, acneform lesions have been induced experimentally by the injection of *P. acnes* into the ears of rats [DeYoung et al., 1984]. The *P. acnes* bacterium is identical to *Corynebacterium parvum*, which has been studied extensively because of its immunoadjuvant properties [Pringle et al., 1982]. Why these organisms should be present in large numbers in man is not known, but a hypothesis has been advanced that they serve an immunosurveillant function [Shuster, 1976].

The follicular microbial flora is comprised principally of *P. acnes* that reside in large numbers deep in the follicles [Leyden, 1983]. Other diphtheroids, aerobic cocci, and yeasts are more superficially located. However, only *P. acnes* is considered to be of pathogenic importance in acne. As mentioned earlier, sebum is the substrate for *P. acnes* growth, and one can demonstrate a positive correlation between the number of *P. acnes* organisms and the quantity of sebaceous gland lipids [McGinley et al., 1980].

In its early stages, inflammation in acne occurs because *P. acnes* elaborates certain chemotactic factors [Puhvel and Sakamoto, 1978] that are able to penetrate the undisrupted (although abnormal) follicular epithelium and attract neutrophils to the follicular site (Fig. 2). Microscopic study of very early acne lesions reveals neutrophils at the follicular site [Kligman, 1974]. Subsequent ingestion of the intrafollicular *P. acnes* organisms by neutrophils releases a variety of hydrolytic enzymes, which then cause a breakdown of the follicle wall, with the release of comedonal contents into the dermis and resultant inflammation.

Once the follicular wall has been breached, the inflammation that results is expressed clinically as inflamed follicular lesions. The *P. acnes* bacteria can activate both the classical and alternative pathways of complement [Webster et al., 1978; Webster and McArthur, 1982]. As a result, C5-derived chemoattractants are generated from neutrophils, further enhancing the inflammatory response. Additional inflammation results from extruded follicular epithelial cells, hair fragments, and lipid components, primarily free fatty acids [Tucker et al., 1980].

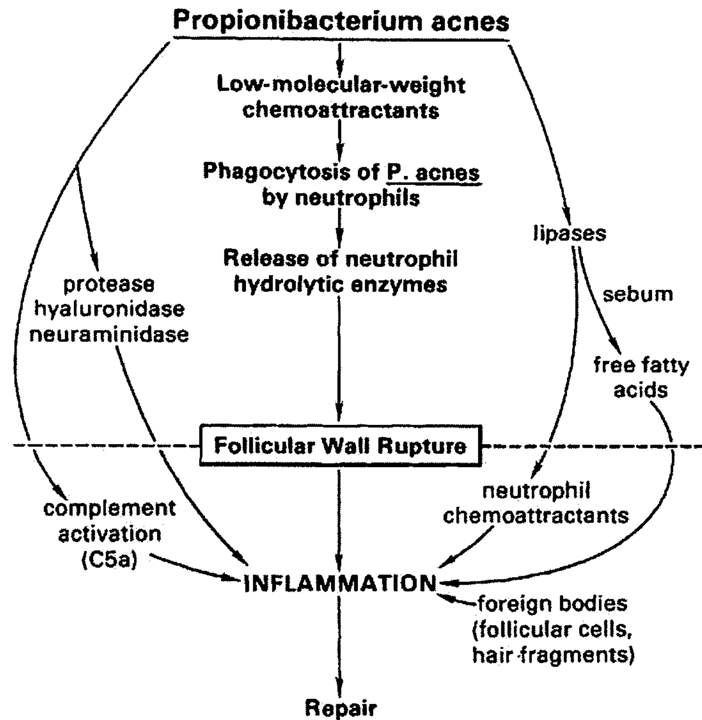

Fig. 2. The known mechanisms by which inflammation occurs in acne, in large measure from the presence of *Propionibacterium acnes*.

Once an inflamed lesion is formed, it generally lasts for 2 to 3 weeks, but there often remains a residual, minimal degree of inflammation for many months or longer [Kligman, 1974]. It is not clear why this inflammation may persist for long periods of time. A possible explanation is that *P. acnes* organisms phagocytosed by neutrophils are not killed readily [Webster et al., 1985]. Their inactivation would take a relatively long time, even when a patient with acne is receiving antibiotic therapy that is successfully preventing the formation of new lesions. Little is known of the role of immunity in the disappearance of inflammation of acne lesions.

Therapeutic Considerations

It seems well established that *P. acnes* elicits much of the inflammation in acne. Modalities that can reduce follicular numbers of the bacterium will aid in preventing the formation of new inflammatory lesions [Stoughton, 1982; Reisner, 1983].

Both oral and topical antibacterial approaches have been used to treat acne. An oral antibiotic is chosen when *P. acnes* is sensitive to it in vitro and when one can demonstrate decreased numbers of bacteria in vivo after the patient is under treatment. Such drugs include tetracycline (and its derivatives), erythromycin, clindamycin, and trimethoprimsulfamethoxazole [Pochi, 1985b]. Two points are worthy of emphasis: 1) some agents, e.g., penicillins and hexachlorophene, are potent inhibitors of *P. acnes* in vitro [Pochi and Strauss, 1961; Denys et al., 1983; Nacht et al., 1981], but are ineffective in vivo, presumably because they are unable to gain sufficient access into the sebaceous follicle; and 2) certain antibiotics, e.g.,

TABLE 2. Topical Antibacterial Agents for Acne

Benzoyl peroxide
Chlorhexidine gluconate
Clindamycin phosphate
Erythromycin
Meclocycline sulfosalicylate
Tetracycline

tetracycline, may have an antiinflammatory effect independent of their antibacterial action [Elewski et al., 1983; Esterly et al., 1984; Miyachi et al., 1986]. This aspect of an antibiotic's action in acne has received limited study.

The use of topical antibacterial and antibiotic compounds is the mainstay in the treatment of most cases of acne, i.e., those with a mild-to-moderate degree of nonscarring, small papular and pustular acne (Table 2). Benzoyl peroxide is generally the most effective; it is presumed to act through the release of nascent oxygen [Nacht et al., 1981], thus reducing the proliferation of the anaerobic *P. acnes* intrafollicular population. The primary irritant effect of benzoyl peroxide, however, together with other problems from its use, such as allergic sensitization, bleaching of colored fabrics, and tumor promotion, renders it less than the ideal agent for topical antibacteriostasis. In the form of washes and soaps, it is even less effective, as the amount of material deposited on the skin after rinsing is far less than that of gels, lotions, and masks, which allow for continuing intrafollicular penetration.

Among the other washes, chlorhexidine gluconate suppresses *P. acnes* in vitro and in one controlled clinical study was shown to be effective in acne [Stoughton and Leyden, 1987]. Similar classes of agents, e.g., alcohol and hexachlorophene, are suppressive of *P. acnes* in vitro but ineffective, on topical usage in vivo, presumably because of inadequate intrafollicular penetration.

Topical antibiotics comprise a generic group that reduce *P. acnes* counts and the activity of acne on epicutaneous administration (Table 2). Studies have disclosed that the two most common topical antibiotics in clinical usage, clindamycin phosphate and erythromycin, reduce the numbers of inflammatory lesions but that the effect is less than that produced by the vehicle itself, including a placebo effect [Dobson and Belknap, 1980; Becker et al., 1981]. As the administration of their oral counterparts achieves a more consistently and quantitatively greater clinical effect on acne, it is presumed that the concentrations reaching the sebaceous follicular site from peroral use are greater, although no comparisons are available to establish whether this is true or not. Inherent in this consideration of the comparative efficacy of topical vs. oral administration of antibiotics is the potentially misleading interpretation of quantitative *P. acnes* inhibition after antibacterial treatment. On the face, the site usually tested for *P. acnes,* the organisms generally range in number from 3–6 log $10/cm^2$. If an individual with a *P. acnes* density of 5 log $10/cm^2$, treated with a topical (or even an oral) antibacterial, shows a 1-log reduction in the numbers of *P. acnes*, this would represent a 90% reduction in the numbers of bacteria. But there would remain 50,000 $organisms/cm^2$. Thus, while a 90% reduction gives the appearance of virtually depleting the flora, there actually are comparatively large numbers of bacteria still present in the follicle. Moreover, the methods of sampling the anaerobic diphtheroids consist largely of the recovery of the superficial portion of the *P. acnes* within the follicle, so that it cannot be stated with certainty how much, if any, of the more deeply resident organisms are affected by topical administration.

In the actual use of antibacterial agents for acne vis-à-vis their *P. acnes*-inhibiting potency in vivo, a minimum 1-log reduction of *P. acnes* is required for improvement to occur. With a 2-log reduction in *P. acnes,* clinical improvement is virtually assured.

CONCLUSIONS

Acne is an inflammatory follicular disorder of the skin. Androgens and bacteria play critical roles in the evolution of the inflammatory lesion, although neither are primary events in the pathologic process. Although models are in place for testing the effect of antiandrogenic modalities to suppress sebum and acne, there is to date no effective topical antiandrogen that can suppress sebaceous gland and acne activity. On the other hand, anti-*P. acnes* topical products are marketed and are clinically useful. In many instances, however, the anti-*P. acnes* inhibition in vivo is only partial, and it is possible that greater penetration of such agents can be achieved for more effective suppression of acne activity.

REFERENCES

Becker, L.E., Bergstresser, P.R., Whiting, D.A., Clendenning, W.E., Dobson, R. L., Jordon, W.P., Abell, E., LeZotte, L.A., Pochi, P.E., Shupack, J.L., Sigafoes, R.B., Stoughton, R.B., and Voorhees, J.J.: Topical clindamycin therapy for acne vulgaris. A cooperative clinical study. Arch. Dermatol. **117**:482–485, 1981.

Bloch, B.: Metabolism, endocrine glands and skin-diseases, with special reference to acne vulgaris and xanthoma. Br. J. Dermatol. **43**:61–87, 1931.

Bruchovsky, N. and Wilson, J.D.: Conversion of testosterone to 5α-androstan-17β-ol-3-one by rat prostate *in vivo* and *in vitro*. J. Biol. Chem. **243**:2012–2021, 1968.

Cunliffe, W.J.: Evolution of a strategy for the treatment of acne. J. Am. Acad. Dermatol. **16**:591–599, 1987.

Cunliffe, W.J. and Shuster, S.: Pathogenesis of acne. Lancet **1**:685–687, 1969.

Cunliffe, W.J., Shuster, S., and Cassels Smith, A.J.: The effect of cyproterone acetate on sebum secretion in patients with acne. Br. J. Dermatol. **81**:200–201, 1969.

Denys, G.A., Jerris, R.C., Swenson, J.M., and Thornsberry, C.: Susceptibility of *Propionibacterium acnes* clinical isolates to 22 antimicrobial agents. Antimicrob. Agents Chemother. **23**:335–337, 1983.

DeYoung, L.M., Young, J.M., Ballaron, S.J., Spires, D.A., and Puhvel, S.M.: Intradermal injection of *Propionibacterium acnes:* A model of inflammation relevant to acne. J. Invest. Dermatol. **83**:394–398, 1984.

Dobson, R.L. and Belknap, B.S.: Topical erythromycin solution in acne. Results of a multiclinic trial. J. Am. Acad. Dermatol. **3**:478–482, 1980.

Downing, D.T., Strauss, J.S., and Pochi, P.E.: Variability in the chemical composition of human skin surface lipids. J. Invest. Dermatol. **53**:322–327, 1969.

Downing, D.T., Stewart, M.E., Wertz, P.W., and Strauss, J.S.: Essential fatty acids and acne. J. Am. Acad. Dermatol. **14**:221–225, 1986.

Downing, D.T., Stewart, M.E., Wertz, P.W., Colton, S.W., Abraham, W., and Strauss, J.S.: Skin lipids: An update. J. Invest. Dermatol. 88(March suppl):2–6, 1987.

Ebling, F.J. and Fanta, D.: Antiandrogens and acne. Semin. Dermatol. **1**:275–284, 1982.

Ebling, F.J. and Skinner J.: The local effects of topically applied estradiol, cyproterone acetate, and ethanol on sebaceous secretion in intact male rats. J. Invest. Dermatol. **81**:448–451, 1983.

Elewski, B.E., Lamb, B.A.J., Sams, W.M. Jr., and Gammon, W.R.: In vivo suppression of neutrophil chemotaxis by systemically and topically administered tetracycline. J. Am. Acad. Dermatol. **8**:807–812, 1983.

Esterly, N.B., Koransky, J.S., Furey, N.L., and Trevisan, M.: Neutrophil chemotaxis in patients with acne receiving tetracycline therapy. Arch. Dermatol. **120**:1308–1313, 1984.

Green, S.G., Stewart, M.E., and Downing, D.T.: Variation in sebum fatty acid composition among adult humans. J. Invest. Dermatol. **83**:114–117, 1984.

Greenwood, R., Brummitt, L., Burke, B., and Cunliffe, W.J.: Treatment of acne with either tetracycline, oestrogen/cyproterone acetate or combined therapy: A double-blind laboratory and clinical study. Br. Med. J. **291**:1231–1235, 1985.

Harris, H.H., Downing, D.T., Stewart, M.E., and Strauss, J.S.: Sustainable rates of sebum secretion in acne patients and matched normal control subjects. J. Am. Acad. Dermatol. **8**:200–203, 1983.

Held, B.L., Nader, S., Rodriguez-Rigau, L.J., Smith, K.D., and Steinberger, E.: Acne and hyperandrogenism. J. Am. Acad. Dermatol. **10**:223–226, 1984.

Hammerstein, J. and Cupceancu, B.: Behandlung des Hirsutismus mit Cyproteronacetat. Dtsch. Med. Wochenschr. **94**:829–834, 1969.

Hammerstein, J., Meckies, J., Leo-Rossberg, I., Moltz, I., and Zielske, F.: Use of cyproterone acetate (CPA) in the treatment of acne, hirsutism and virilism. J. Steroid Biochem. **6**:827–836, 1975.

Hellgren, L., Puschmann, M., and Vincent, J.: Simultaneous determination of lipids from skin and hair after exposure to topical chlormadinone acetate. Arch. Dermatol. Res. **274**:369–371, 1982.

Kligman, A.M.: The uses of sebum? In Montagna W., Ellis R.A., and Silver, A.F. (eds): "Advances in Biology of Skin" (Vol. 4). Oxford: Pergamon Press, 1963, pp. 100–124.

Kligman, A.M.: An overview of acne. J. Invest. Dermatol. **62**:268–287, 1974.

Knutson, D.D.: Ultrastructural observations in acne vulgaris: The normal sebaceous follicle and acne lesion. J. Invest. Dermatol. **62**:288–307, 1974.

Kraning, K.K. and Odland, G.F.: Analysis of research needs and priorities in dermatology. J. Invest. Dermatol. **73**:434–442, 1979.

Lavker, R.M., Leyden, J.J., and McGinley, K.J.: The relationship between bacteria and the abnormal follicular keratinization in acne vulgaris. J. Invest. Dermatol. **77**:325–330, 1981.

Leyden, J.J.: Follicular microflora in acne vulgaris. Dermatol. Clin. **1**:345–351, 1983.

Leyden, J.J., McGinley, K.J., Mills, O.H., and Kligman, A.M.: Age-related changes in the resident bacterial flora of the human face. J. Invest. Dermatol. **65**:379–381, 1975.

Lookingbill, D.P., Horton, R., Demers, L.M., Egan, N., Marks, J.G., Jr., and Santen, R.J.: Tissue production of androgens in women with acne. J. Am. Acad. Dermatol. **12**:481–487, 1985.

Lovejoy, E.D. and Hastings, T.W.: Isolation and growth of the acne bacillus. J. Cutan. Dis. **29**:80, 1911.

Lucky, A.W.: Topical antiandrogens. What use in dermatology? Arch. Dermatol. **121**:55–56, 1985.

Lucky, A.W., McGuire, J., Rosenfield, R.L., Lucky, P.A., and Rich, B.H.: Plasma androgens in women with acne. J. Invest. Dermatol. **81**:70–74, 1983.

Lyons, F. and Shuster, S.: Sex difference in response of the human sebaceous gland to topical flutamide. Br. J. Dermatol. **107**:697–699, 1982.

Marynick, S.P., Chakmakjian, Z.H., McCaffree, D., and Herndon, J.H.: Androgen excess in cystic acne. N. Engl. J. Med. **308**:981–986, 1983.

McGinley, K.J., Webster, G.F., and Leyden, J.J.: Regional variations of cutaneous propionibacteria. Appl. Env. Microbiol. **35**:62–65, 1978.

McGinley, K.J., Ruggieri, M., Webster, G.F., and Leyden, J.J.: Regional variations in density of cutaneous Propionibacteria: Correlation of *P. acnes* populations with sebaceous secretion. J. Clin. Microbiol. **12**:672–675, 1980.

Miyachi, Y., Yoshioka, A., Imamura, S., and Niwa, Y.: Effect of antibiotics on the generation of reactive oxygen species. J. Invest. Dermatol. **86**:449–453, 1986.

Morello, A.M., Downing, D.T., and Strauss, J.S.: Octadecadienoic acids in the skin surface lipids of acne patients and normal subjects. J. Invest. Dermatol. **66**:319–323, 1976.

Nacht, S., Yeung, D., Beasley, J.N., Anjo, M.D., and Maibach, H.I.: Benzoyl peroxide: Percutaneous penetration and metabolic disposition. J. Am. Acad. Dermatol. **4**:31–37, 1981.

Nader, S., Gans, E.H., McGinley, K.J., and Kligman, A.M.: Comparative activity of benzoyl peroxide and hexachlorophene. Arch. Dermatol. **119**:577–579, 1983.

Nader, S., Rodriguez-Rigau, L.J., Smith K.D., and Steinberger, E.: Acne and hyperandrogenism: Impact of lowering androgen levels with glucocorticoid treatment. J. Am. Acad. Dermatol. **11**:256–259, 1984.

Neumann, F.: Pharmacological basis for clinical use of antiandrogens. J. Steroid Biochem. **19**:391–402, 1983.

Neumann, F. and Töpert, M.: Pharmacology of antiandrogens. J. Steroid Biochem. **25**:885–895, 1986.

Pochi, P.E.: Androgenic effects on human sebaceous glands. Arch. Dermatol. **118**:803–804, 1982a.

Pochi, P.E.: Hormones and acne. Semin. Dermatol. **1**:265–273, 1982b.

Pochi, P.E.: The sebaceous gland. In Maibach H.I. and Boisits, E.K., (eds): "Neonatal Skin: Structure and Function." New York: Marcel Dekker, Inc., 1982c, pp. 67–80.

Pochi, P.E.: Hormonal therapy of acne. Dermatol. Clin. **1**:377–384, 1983.

Pochi, P.E.: Pathogenesis of acne. Importance of follicular epithelial changes. In Epstein, E. (ed): "Controversies in Dermatology." Philadelphia: WB Saunders Co., 1984a, pp. 256–258.

Pochi, P.E.: Effect of hormonal treatment regimens on sebum and acne. Clin. Res. **32**:714A, 1984b.

Pochi, P.E.: Acne vulgaris. In Demis, D.J. (ed): "Clinical Dermatology." Harper and Row: Philadelphia, 1985a.

Pochi, P.E.: Management of refractory acne. Hosp. Pract. **20**:73–82, 1985b.

Pochi, P.E. and Strauss, J.S.: Antibiotic sensitivity of *Corynebacterium acnes (Propionibacterium acnes)*. J. Invest. Dermatol. **36**:423–429, 1961.

Pochi, P.E. and Strauss, J.S.: Sebaceous gland suppression with ethinyl estradiol and diethylstilbestrol. Arch. Dermatol. **108**:210–214, 1973.

Pochi, P.E. and Strauss, J.S.: Endocrinologic control of the development and activity of the human sebaceous gland. J. Invest. Dermatol. **62**:191–201, 1974.

Pochi, P.E. and Strauss J.S.: Sebaceous gland inhibition from combined glucocorticoid-estrogen treatment. Arch. Dermatol. **112**:1108–1109, 1976.

Pochi, P.E., Strauss, J.S., and Downing, D.T.: Skin surface lipid composition, acne, pubertal development, and urinary excretion of testosterone and 17-ketosteroids. J. Invest. Dermatol. **69**:485–489, 1977.

Pochi, P.E., Strauss, J.S., and Downing, D.T.: Age-related changes in sebaceous gland activity. J. Invest. Dermatol. **73**:108–111, 1979.

Pochi, P.E., Leyden, J.J., Shalita, A.R., and Strauss, J.S.: "Acne. Current Concepts." Kalamazoo: The Upjohn Company, 1981, pp. 3–27.

Pringle, A.T., Cummings, C.S., Bishop, B.F., and Viers, V.S.: Fate of vaccines of *Propionibacterium acnes* after phagocytosis by murine macrophages. Infect. Immunol. **38**:371–374, 1982.

Puhvel, S.M.: *Propionibacterium acnes* and acne vulgaris. Semin. Dermatol. **1**:293–298, 1982.

Puhvel, S.M. and Sakamoto, M.: The chemoattractant properties of comedonal contents. J. Invest. Dermatol. **71**:324–329, 1978.

Pye, R.J., Burton, J.L., and Harris, J.I.: Effect of 1% cyproterone acetate in Cetomacrogol cream BPC (formula A) on sebum excretion rate in patients with acne. Br. J. Dermatol. **95**:147–148, 1976.

Rebello, T.J. and Hawk, L.M.: Skin surface glycerol levels in acne vulgaris. J. Invest. Dermatol. **70**:353–354, 1978.

Reisner, R.M.: Antibiotic and anti-inflammatory therapy of acne. Dermatol. Clin. **1**:385–397, 1983.

Sabouraud, R.: La séborrhée grasse et la pelade. Ann. Inst. Pasteur. **11**:134–159, 1897.

Saihan, E.M. and Burton, J.L.: Sebaceous gland suppression in female acne patients by combined glucocorticoid-oestrogen treatment. Br. J. Dermatol. **103**:139, 1980.

Sansone, G. and Reisner, R.M.: Differential rates of conversion of testosterone to dihydrotestosterone in acne and in normal human skin—a possible pathogenic factor in acne. J. Invest. Dermatol. **56**:366–372, 1971.

Schmidt, J.B. and Spona, J.: Ein wirksames topisches Antiandrogen-17α-Propylmesterolon-bei Akne. Hautarzt **38**:470–473, 1987.

Serri, F. and Huber, W.M.: The development of sebaceous glands in man. In Montagna, W., Ellis, R.A., and Silver, A.F. (eds): "Advances in Biology of Skin" (Vol. 4). Oxford: Pergamon Press, 1963, pp. 1–18.

Shalita, A.R., Leyden, J.J., Pochi, P.E., and Strauss, J.S.: Acne vulgaris. J. Am. Acad. Dermatol. **16**:410–412, 1987.

Shuster, S.: Biological purpose of acne. Lancet **1**:1328, 1976.

Simpson, N.B., Bowden, P.E., Forster, R.A., and Cunliffe, W.J.: The effect of topically applied progesterone on sebum excretion rate. Br. J. Dermatol. **100**:687–692, 1979.

Stewart, M.E. and Downing, D.T.: Proportions of various straight and branched fatty acid chain types in the sebaceous wax esters of young children. J. Invest. Dermatol. **84**:501–503, 1985.

Stewart, M.E., Grahek, M.O., Cambier, L.S., Wertz, P.W., and Downing, D.T.: Dilutional effect of increased sebaceous gland activity on the proportion of linoleic acid in sebaceous wax esters and in epidermal acylceramides. J. Invest. Dermatol. **87**:733–736, 1986.

Stoughton, R.B.: Topical antibiotics and acne. Semin. Dermatol. **1**:251–256, 1982.

Stoughton, R.B. and Leyden, J.J.: Efficacy of 4 percent chlorhexidine gluconate skin cleanser in the treatment of acne vulgaris. Cutis **39**:551–553, 1987.

Strauss, J.S. and Pochi, P.E.: The human sebaceous gland: Its regulation by steroidal hormones and its use as an end-organ for assaying androgenicity *in vivo*. Recent Prog. Horm. Res. **19**:385–444, 1963.

Strauss, J.S. and Pochi, P.E.: Systemic estrogen therapy of acne. Progr. Dermatol. **2**:7–10, 1967.

Strauss, J.S. and Pochi, P.E.: Assay of anti-androgens in man by the sebaceous gland response. Br. J. Dermatol. **82(suppl 6)**:33–42, 1970.

Tamm, J., Seckelmann, M., Volkwein, U., and Ludwig, E.: The effect of the antiandrogen 11α-hydroxyprogesterone on sebum production and cholesterol concentration of sebum. Br. J. Dermatol. **107**:63–70, 1982.

Tucker, S.B., Rogers, R.S., Winkelmann, R.K., Privett, O.S., and Jordon, R.E.: Inflammation in acne vulgaris. Leukocyte attraction and cytotoxicity by comedonal material. J. Invest. Dermatol. **74**:21–25, 1980.

Unna, P.G.: "The Histopathology of the Diseases of the Skin," translated by N. Walker. New York: Macmillan & Co., 1896, pp. 352–366.

Walton, S., Cunliffe, W.J., Lookingbill, P., and Keczkes, K.: Lack of effect of topical spironolactone on sebum excretion. Br. J. Dermatol. **114**:261–269, 1986.

Webster, G.F. and McArthur, W.P.: Activation of components of the alternative pathway of complement by *Propionibacterium acnes* cell wall carbohydrate. J. Invest. Dermatol. **79**:137–140, 1982.

Webster, G.F., Leyden, J.J., Norman, M.E., and Nilsson, U.R.: Complement activation in acne vulgaris: In vitro studies with *Propionibacterium acnes* and *Propionibacterium granulosum.* Infect. Immunol. **22**:523–529, 1978.

Webster, G.F., Ruggieri, M.R., and McGinley, K.J.: Correlation of *Propionibacterium acnes* populations with the presence of triglycerides on nonhuman skin. Appl. Env. Microbiol. **41**:1269–1270, 1981.

Webster, G.F., Leyden, J.J., Musson, R.A., and Douglas, S.D.: Susceptibility of *Propionibacterium acnes* to killing and degradation by human neutrophils and monocytes in vitro. Infect. Immunol. **49**:116–121, 1985.

Wessler, S.: Thrombosis and sex hormones: A perplexing liaison. J. Lab. Clin. Med. **96**:757–761, 1980.

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EXHIBIT 2

Classic diseases revisited

Acne: a review of immunologic and microbiologic factors

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Summary
Acne vulgaris is a self-limiting skin disorder seen primarily in adolescents, whose aetiology appears to be multifactorial. The four main aetiological factors are hypercornification of the pilosebaceous duct, increased sebum production, colonization with *Propionibacterium acnes*, and subsequently the production of inflammation. Considerable investigation has addressed the immunologic reaction to extracellular products produced by the acne-causing organism, *P acnes*. The immunologic response involves both humoral and cell-mediated pathways. Further research should clarify the role of complement, cytotoxins, and neutrophils in this acne-forming response.

Keywords: acne vulgaris; *Propionibacterium acnes*

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Acne vulgaris, the most common cutaneous disorder, is manifested by comedones, papules, pustules, and cysts. The aetiology of acne appears to be multifactorial. The exact mechanism triggering the development of the comedone and the stimuli causing the non-inflamed lesion to become inflamed are poorly understood. The microbiology of acne vulgaris and its immunologic ramifications constitute the major thrust of present research in the elucidation of the pathogenesis of the inflammatory acne lesion.

The microbiology of the pilosebaceous unit involves three coexisting groups of microorganisms: Gram-positive, coagulase-negative cocci (staphylococci and micrococci); anaerobic diphtheroids (*Propionibacterium acnes* and *Propionibacterium granulosum*); and lipophilic yeasts (*Pityrosporum* species). The microflora of comedones is qualitatively identical to that of the normal sebaceous follicle.

The staphylococci and micrococci are aerobes; therefore, their site of growth within the sebaceous unit is superficial, and these organisms are unable to reside in the anaerobic conditions of the infra-infundibulum where the inflammatory reaction occurs in acne. Antibiotics that selectively eliminate these organisms do not affect the clinical response of patients,[1] and their role in acne pathogenesis is negligible.

The lipophilic yeasts that reside in hair follicles have been divided morphologically into *Pityrosporum ovale* and *Pityrosporum orbiculare*. These represent a complex of organisms and have been reclassified as *Malassezia furfur* as well as other *Malassezia* species.[2] The filamentous forms that are usually associated with a pathologic condition are not seen in acne lesions and, save for tinea versicolor, these organisms appear not to play a significant aetiologic role in any disease state.

If the microbial flora is significant in the pathogenesis of acne, the most likely organism to blame is *P acnes*, a strict anaerobe that has been shown serologically and biochemically to be identical to *Corynebacterium parvum*, a potent stimulator of the reticuloendothelial system.[3] This organism has been used as an immunostimulatory adjunct in chemotherapy of numerous tumours.[3-6] *P acnes* is overwhelmingly the predominant microorganism in the normal pilosebaceous follicle, as well as in the acne state, and has been divided into two serotypes and five biotypes. Up to 10^7 viable *P acnes* have been isolated from a single sebaceous unit. *P acnes* is not pathogenic by normal standards because there is no correlation between the number of bacteria and the severity and type of acne. Nevertheless, *P acnes* appears to be the target of oral and topical antibiotic usage, and the reduction in numbers of *P acnes* is a just parameter of therapeutic effectiveness of antibiotics.

P acnes secretes several extracellular products that may be significant in the aetiology of acne. These include hyaluronidase, proteases, lipases, and chemotactic factors for neutrophils, lymphocytes, and macrophages. The microenvironment of the pilosebaceous unit is likely to play a major role in the amount of exoenzymes that are produced by the organism because *in vitro* studies demonstrate that their production is altered by factors such as pH and oxygen tension.

The current interest in *P acnes* revolves around whether its immunopotentiating properties are pertinent in the pathogenesis of acne. Specifically, there is reason to believe that *P acnes* may be a direct instigator of inflammation in acne via its interaction with antibody and complement, its chemotactic properties, and via cell-mediated immunity.

Humoral immunity

Patients with inflammatory acne develop an immune response to *P acnes*. Circulating immune complexes have been reported to be elevated in some acne patients. The degree of elevation has been correlated with the severity of acne inflammation.[7,8] Additionally, complement-fixing antibody titres to *P acnes* are

elevated, and the titres parallel the severity of the inflammation.[9] In minimal acne, these antibody titres are rarely greater than the levels found for most adults. The antibodies to *P acnes* have not been characterised fully, although they are reported to be largely of the IgG class. Total IgG levels are slightly increased in some patients with severe acne, which may reflect an enhanced B-cell activity,[10] although they may be lower than normal in others.[11] Titres of IgG3 have been demonstrated to be higher in severe cases.[12 13] We have shown the dominant antigen to be in the soluble extract of *P acnes* and to have a carbohydrate component.[14]

The elevated antibody response to *P acnes* appears to be specific because antibody titres for *Staphylococcus epidermidis* are not raised in acne. At least four major antigenic components have been detected on analysis of the extracellular supernatant fluid from dialysed *P acnes* cultures.[15] Variations in the antigenic composition of *P acnes* may account for some of the differences in antibody patterns that are seen in individuals.

Immunofluorescence studies have revealed *P acnes* antigens in the dermis surrounding the pilosebaceous units in acne patients. In contrast, the antigens are confined totally within the follicular walls in normal skin.[16]

Cell-mediated immunity

Cell-mediated immunity could contribute to the development of inflammation in acne; however, this role is far from having been proven. Skin tests, made with common recall antigens such as trichophytin, mumps, or purified protein derivative, have demonstrated that patients with severe acne may have a depressed or absent reactivity.[17] In addition, sensitisation to dinitrochlorobenzene may not occur.[17] Such deficiencies are not matched by defects in mitogen-induced lymphocyte blastogenesis *in vitro* where the responses of patients' lymphocytes to phytohaemagglutinin occur at normal levels.[17–19] However, acne patients have a depressed number of E-rosette-forming cells, indicating that some form of T-cell deficiency may be present.[11]

Specific responses to antigens prepared from *P acnes* have been studied. Various types of skin test responses are reported, including both the classic immediate-type and delayed-type hypersensitivity reactions, as well as ill-defined erythematous reaction that disappears before 48 hours postinjection.[20] The latter may be caused by the inflammation brought about from the activation of complement by antibody–antigen complexes or by materials in the antigen preparation.

The type of skin test response shown by acne patients appears to depend, at least in part, on the nature of the antigen preparation, the dosage used for the skin test, and possibly on the isolate of *P acnes* from which the antigen is prepared. Thus, Puhvel *et al*[20] reported immediate hypersensitivity reactions as being characteristic of acne patients who had been skin-tested with *P acnes* antigen prepared from disrupted cells or from a dialyzed culture filtrate. Less than half of their patients developed a delayed reaction that remained visible at 48 hours. In contrast, Kersey *et al*[21] used heat-killed *P acnes* as antigen and reported strong delayed responses in the patients, the strongest response being found for the most severe acne cases. It is a distinct possibility that the strong immediate skin test response reported by Puhvel *et al*[20]prevented subsequent development of a delayed response, a phenomenon that was described over 40 years ago for the trichophytin skin test.

Although the results from specific skin tests are still somewhat confusing, it is generally accepted that the *in vitro* tests for lymphocyte transformation and for production of the lymphokine leukocyte migration inhibitory factor show that acne patients' lymphocytes develop a hyperreactivity to *P acnes* antigens.[19 22] Thus, the stage is set for a contribution by cell-mediated immune responses to the inflammation in acne.

Complement activation

The activation of complement leads to the release of inflammatory mediators, causing mast cell degranulation, leukocyte chemotaxis, and lysosomal enzyme release. Both comedonal contents and *P acnes* have been shown to activate complement via both the classic and the alternate pathways.[23]

Immunofluorescence of skin specimens from acne patients have revealed the presence of C3 deposits in the dermal vessel walls.[24] On occasion, immunoglobulins are seen in addition to complement, and this provides suggestive evidence of the formation of immune complexes around the acne lesion. Thus, complement fixation may play a major role in inducing the inflammation seen in the acne lesion.

Cytotoxins and neutrophil function

Early acne lesions reveal polymorphonuclear leukocytes accumulating at the periphery of pilosebaceous units and later migrating within the hair follicle. The production of cytotoxins, which stimulate chemotactic activity independently of complement, has been investigated in acne. Materials produced by *P acnes* and by other comedonal bacteria grown *in vitro* can stimulate neutrophil chemotaxis.[25] The fraction of *P acnes* with chemotactic activity appears to consist of predominantly low molecular weight material.[26] The lipid-containing fraction extracted from comedones can induce neutrophil chemotaxis.[27] However, crude comedonal extracts are reported to be toxic for neutrophils, with the free fatty acids appearing to be responsible for the cytotoxicity.[28] The crude comedonal extract is, however, a chemoattractant for monocytes, even though toxicity is reported.[28] Thus, the importance of cytotoxin production for acne lesion formation is unclear at present. Indeed, the activation of complement by both comedonal material and *P acnes* may be of greater importance for inducing neutrophil chemotaxis in acne.

There have been relatively few studies on neutrophil function in acne patients. Enhanced chemotactic and random migratory activities have been reported by some,[29] but others find activity at normal levels.[30] In general, phagocytic activities appear normal for bacteria such as *Staphylococcus aureus*.[30] Lee and Shalita[10] have indicated that some patients with severe acne have a marked depression in their neutrophil chemotaxis, while in other patients chemotaxis is increased. In this latter group, they reported finding a defect in phagocytosis that was specific for *P acnes*. Further research should clarify the role of abnormal neutrophil functions in the pathogenesis of acne.

Treatment considerations

Acne therapy must address the aetiological factors involved in acne pathogenesis. These treatment considerations include correcting the altered pattern of follicular keratinization, reducing sebaceous gland production, diminishing the *P acnes* population in the follicle and inhibiting its production of extracellular inflammatory products, and producing an anti-inflammatory effect.

Coexistent with the immune theories of the pathogenesis of acne, there have been several modes of therapy applied to patients with severe acne. Possibly because of the presence of isotretinoin or the venerable microcomedonal theory of acne, few clinical trials have materialised since the early 1980s.

Intralesional and oral steroids are occasionally used to reduce inflammation in severe cases of acne. Cimetidine, which affects cell-mediated immunity as well as being anti-androgenic, has had conflicting results as to its therapeutic efficacy.[31] Levamisole restored the impaired T-cell function in acne patients as well as bringing about some clinical improvement.[11] A polyvalent *P acnes* vaccine has been reported to have had modest success,[32] as has transfer factor.[33] Finally, it has been suggested that tetracycline, which becomes concentrated in inflamed lesions and has been the mainstay of acne treatment for two decades, could act by inhibiting neutrophil chemotaxis rather than its antibacterial actions.[29]

P acnes plays a central role in acne pathogenesis. Not only does this anaerobic bacterium produce lipases, proteases, and other extracellular enzymes, it also secretes chemotactic factors attracting polymorphonuclear leukocytes, lymphocytes, and macrophages. The inflammatory response initiated by these extracellular products stimulates the classical and alternative complement pathways and other immune respones. Thus, *P acnes* directly contributes to the existence of acne via its effects on humoral and cell-medicated immunity, complement activation, and cytotoxin production. Further studies on the immunological factors involved in acne pathogenesis are warranted.

1 Plewig G, Kligman AM. *Acne*. New York, Springer-Verlag, 1975; pp 38–41.
2 Gueho E, Boekhout T, Ashbee HR, Guillot J, Van Belkum A, Faergemann J. The role of *Malassezia* species in the ecology of human skin and as pathogens. *Med Mycol* 1998;**36**(suppl 1):220–9.
3 Adlam C, Scott MT. Lymphoreticular stimulatory properties of *Corynebacterium parvum* and related bacteria. *J Med Microbiol* 1973;6:261–5.
4 Senaldi G, Yin S, Shaklee CL, Piguet P, Mak TW, Ulich TR. *Cornyebacterium parvum* and *Mycobacterium bovis* Bacillus Calmette-Guerin-induced granuloma formation is inhibited in TNF Receptor I Knockout Mice and by treatment with soluble TNF-RI. *J Immunol* 1996;157:5022–6.
5 Herberman RB. Natural killer cells and their possible roles in resistance against disease. *Clin Immunol Rev* 1981;1:1–14.
6 Cantrell JL, Wheat RW. Antitumor activity and lymphoreticular stimulation properties of fractions isolated from *Corynebacterium parvum*. *Cancer Res* 1979;39:3554–5.
7 Woolfson H. Acne fulminans with circulating immune complexes and leukaemoid reaction treated with steroids and azathioprine. *Clin Exp Dermatol* 1987;12:463–6.
8 Kellett JK, Beck MH, Chalmers RJ. Erythema nodosum and circulating immune complexes in acne fulminans after treatment with isotretinoin [letter]. *BMJ* 1985;290:820.
9 Puhvel SM, Hoffman K, Sternberg TH. *Corynebacterium acnes*. Presence of complement fixing antibodies to *Corynebacterium acnes* in the sera of patients with acne vulgaris. *Arch Dermatol* 1966;93:364–8.
10 Lee WI, Shalita AR. Leukocyte abnormalities in acne conglobata. *J Invest Dermatol* 1980;74: 258–62.
11 DeCree J, DeCock W, Verhaegen H. Levamisole treatment of inflammatory acne. Restoration of impaired T-cell function accompanied by clearance of the lesions. *Biomedicine* 1979;31:95–8.
12 Holland DB, Ingham E, Gowland G, Cunliffe WJ. IgG subclasses in acne vulgaris. *Br J Dermatol* 1986;114:349–51.
13 Ashbee HR, Muir SR, Cunliffe WJ, Ingham E. IgG subclasses specific to *Staphylococcus epidermidis* and *Propionibacterium acnes* in patients with acne vulgaris. *Br J Dermatol* 1997;136;730–3.

14 Lehmann PF, Burkhart CG, Cantrell JL, Butcher CL. *Propionibacterium acnes* : interaction with complement and development of an enzyme-linked immunassay for the detection of antibody. *Int J Dermatol* 1999; in press.
15 Puhvel SM. Acne from an immunological perspective. *Cutis* 1976;17:502–4.
16 Imamura S, Pochi PE, Strauss JS, McCabes WR. The localization and distribution of *Corynebacterium acnes* and its antigens in normal skin and in lesions of acne vulgaris. *J Invest Dermatol* 1969;53:143–8.
17 Palatsi R. Delayed hypersensitivity and febrile acne conglobata. *Acta Dermatovener* 1977;57: 51–2.
18 Rajka G, Froland S. Lecture at the XI Scand Congr Allerology, Helsinki, May 25, 1973, quoted in Fajka G: On cell-mediated immunity in acne conglobata. *Acta Dermatovener* 1977;57; 141.
19 Puhvel SM, Amirian D, Weintraub J, Reisner RM. Lymphocyte transformation in subjects with nodulocystic acne. *Br J Dermatol* 1977;97: 205–7.
20 Puhvel SM, Hoffman IK, Reisner RM, Sternberg TH. Delayed hypersensitivity of patients with acne vulgaris to *Corynebacterium acnes*. *J Invest Dermatol* 1967;49:154–8.
21 Kersey P, Sussman M, Dahl M. Delayed skin test reactivity to *Propionibacterium acnes* correlates with severity of inflammation in acne vulgaris. *Br J Dermatol* 1980;**103**:651–6.
22 Gowland G, Ward RM, Holland KT, Cunliffe WJ. Cellular immunity to *P acnes* in the normal population and patients with acne vulgaris. *Br J Dermatol* 1978;99:43–5
23 Webster GF, Leyden JJ, Norman ME, Nilsson UR. Complement activation in acne vulgaris: in vitro studies with *Propionibacterium acnes* and *Propionibacterium granulosum*. *Infect Immun* 1978;22:523–6.
24 Dahl MGC, McGibbon DH. Complement in inflammatory acne vulgaris. *BMJ* 1976;2:1383–4.
25 Puhvel S, Sakamoto MA. Cytotoxin production by comedonal bacteria (*Propionibacterium acne, Propionibacteriun granulosum*, and *Staphylococcus epidermidis*). *J Invest Dermatol* 1980;74:36–40.
26 Webster GF, Leyden JJ. Characterization of *Propionibacterium acnes* chemotactic factor. *J Invest Dermatol* 1980;74:254–6.
27 Puhvel S, Sakamoto M. The chemoattractant properties of comedonal components. *J Invest Dermatol* 1978;71:324–6.
28 Tucker SB, Rogers RS III, Windelmann RK. Inflammation in acne vulgaris: leukocyte attraction and cytotoxicity by comedonal materials. *J Invest Dermatol* 1980;74:21–6.
29 Gould DJ, Gowland G, Cunliffe WJ. An investigation of leukocyte function in acne vulgaris. *Br J Dermatol* 1978;99(suppl 16):17–21.
30 Rebora A, Dallegri F, Patrone F. Neutrophil functions in acne conglobata. *Dermatologica* 1979;**159**:217–20.
31 Lyons F, Cook J, Shuster S. Inhibition of sebum excretion by a H_2 blocker. *Lancet* 1979;1:1376.
32 Goldman L, Michael JG, Riebel S. The immunology of acne: a polyvalent *Propionibacteria* vaccine. *Cutis* 1979;23:181–3.
33 Grohn P, Kuokkanen K, Krohn K. The effect of transfer factor on cystic acne. *Acta Dermatovener* 1978;58:153–6.

 EXHIBIT 3 Taylor&Francis healthsciences

REVIEW

Pathological Mechanisms of Acne with Special Emphasis on *Propionibacterium acnes* and Related Therapy

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Acne is a common disease that in cases of extreme disfiguration can have severe consequences for the personality development of young people and is associated with a relatively high prevalence of depression and suicide. Spontaneous regression is common, but acne can extend into the fourth and fifth decades of life. The pathogenesis is still not fully understood. Factors promoting the development of acne are: increased sebum production, ductal cornification, bacterial colonization of the pilosebaceus ducts and inflammation. However, there is evidence that inflammation is not a factor but rather a consequence of the interaction of the other three factors. *Propionibacterium acnes* releases pro-inflammatory cytokines as well as antigens and mitogen(s), with cellular and non-cellular responses to these products triggering inflammation. Treatment is often frustran. Therapeutical strategies are needed based on new understandings of the pathomechanisms involved in acne. The aim of this review is to summarize the data on aetiopathological factors in acne and their contribution to acne pathology and therapy.
Key words: acne therapy; acne vulgaris; mitogen; Propionibacterium acnes.

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Acne vulgaris is one of the most common diseases of the skin and in cases of extreme disfiguration can sometimes have severe consequences for the personality development of young people, with ensuing social and economic problems. Adolescents suffering from acne show higher levels of anxiety and greater social inhibition and aggression compared to non-affected individuals. Among skin diseases, acne vulgaris is the second highest cause of suicides (1).

Acne is an exclusively human disease and a unique condition of human sebaceous follicles of the face, chest and back that begins in the prepubertal child. Spontaneous regression is common, but in about 5% of cases acne persists beyond the age of 25 and extends into the fourth and fifth decades of life (2). The earlier the symptoms start, the more severe is the course of the disease. The prevalence of the disease does not reflect any preference for male or female, but usually the course is more severe in males. There seems to be a familial trait with an autosomal-dominant mode of inheritance with different expression.

AETIOLOGY OF ACNE

Factors promoting the development of acne are: increased sebum production, ductal cornification, bacterial colonization of the pilosebaceus ducts and inflammation (Fig. 1). Although the severity of acne vulgaris is associated with seborrhoea, the disease is one of the follicular infundibulum. In mild acne, the keratinocytes of the infundibulum hypercornify, hyperkeratinize and hypodesquamate to produce comedones. In severe acne the infundibulum ruptures to introduce sebum into the dermis, where it is highly inflammatory.

Sebum production and androgens

Seborrhoea is significantly more common in patients with acne than in controls and contributes to lesion formation (3, 4). The sebaceous gland is an androgen target organ, stimulated to produce sebum at puberty and beyond by androgens. Sebaceous glands present the highest androgen receptor density in human skin (5, 6). The most important androgen is testosterone, which is converted to the more potent dihydrotestosterone (DHT) by the iso-enzyme 5α reductase (type I), the major isotype detected in skin, particularly in sebaceous gland-rich areas (7–10). From cell-culture experiments there is evidence that human sebocytes possess a complete corticotropin-releasing hormone (CRH)-receptor system. CRH is a coordinator for neuroendocrine and behavioural responses to stress. It has been concluded that CRH may function as an important autocrine hormone with a homeostatic pro-differentiation activity (11). Clinical observations suggest an influence of stress on the course of acne, which may be explained via this hormonal pathway.

Fatty acids

Free fatty acids are known to be highly inflammatory and chemotactic (Fig. 1). Exclusive production of irritant fatty acids by the lipases of *P. acnes* acting

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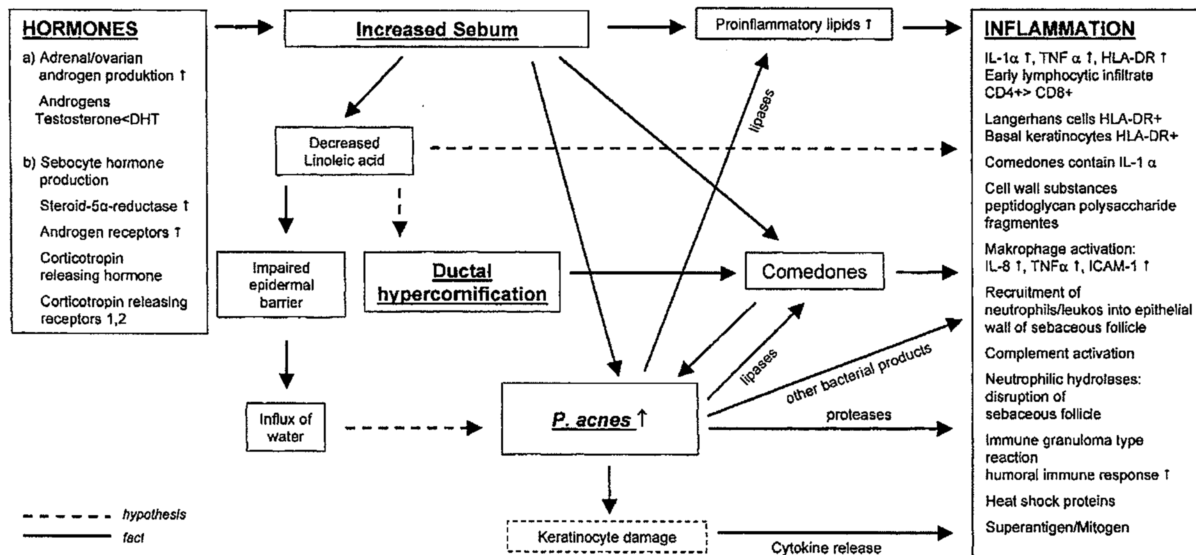

Fig. 1. Synopsis of factors involved in the pathogenesis of acne.

on triglycerides derived from sebocytes was an early hypothesis dashed by Weeks et al. (12), who showed that lipase inhibition induced a reduction of fatty acids on the skin, but failed to treat acne. Pro-inflammatory lipid fractions in sebum produced by other mechanisms than bacterial lipases may be one responsible factor for the development of inflammation in acne.

Linoleic acid deficiency

Linoleic acid is incorporated into sphingolipids in the follicular epithelium, which participates in the formation of the intracellular lipid lamellae. In 1986, it was suggested that linoleic acid deficiency was an important factor in the aetiology of acne (Fig. 1) (13). It was argued that linoleic acid deficiency impaired the follicular epithelium barrier, allowing other free fatty acids, resulting from bacterial lipase activity and/or sebocyte metabolism to enter the epithelium and to induce localized essential fatty acid deficiency. Zouboulis (14) recently showed that linoleic acid is able to regulate interleukin (IL-)8 secretion and, as a consequence, the inflammatory reaction.

Ductal hypercornification

The mechanisms underlying the infundibular changes are still obscure. Prominent hypotheses implicate a local follicular deficiency of linoleic acid, effects of IL-1α and androgens as potential factors involved in follicular hyperkeratinization causing an apparent early cornification of the keratinocytes and scaling (Fig. 1) (15, 16). Il-1α causes an upregulation of cellular retinoic acid binding protein-II and small proline-rich protein 1 in keratinocytes cultures which correlates with keratinocyte differentiation (17).

Oxygen stress and free radicals

Another hypothesis focuses on the importance of reactive oxygen species as an inflammatory mediator released by phagocytes such as neutrophils, which produce these mediators for lysis of invading microorganisms. In 1998, Akamatsu & Horio showed that neutrophil-derived reactive oxygen species are involved in the irritation and destruction of the follicular wall in acne patients (18).

Bacteria

Acne is not an infectious disease and, therefore, not contagious. Among the bacteria species that colonize normal skin as resident flora, only those able to colonize the follicular duct and multiply there can be pathogenic for acne. Only three species of microorganisms could therefore be associated with the development of acne lesions: propionibacteria, coagulase-negative staphylococci, and yeasts of the species *Malassezia*. However, acne did not improve after antifungal treatment, so yeasts could not be associated with the pathogenesis of acne. Staphylococci could also be excluded, because these develop antibiotic resistance during the first weeks of treatment in most patients (19), and the numbers quickly rise. Scientific interest has therefore been focused on propionibacteria.

Propionibacteria are Gram-positive, non-motile, pleomorphic rod-shaped cells that ferment sugars to yield propionic acid as one of the end products in this metabolic process. *P. acnes* is the predominant resident

microorganism on sebaceous gland-rich areas of skin in adults (20). On human skin, propionibacteria can be found from birth until death (21). Bacteriological analysis and sebum production investigated in multiple body areas demonstrated a high association between *P. acnes* levels and sebum production (22).

P. acnes is associated with the development of acne but reports are increasing in number implicating *P. acnes* in other diseases (reviewed in 23) (Table I).

The pathogenicity of propionibacteria is thought to be due to, first, the production of exocellular enzymes and other bioactive exocellular products, which could act as virulence determinants, and, second, on the microorganism's interaction with the immune system. Propionibacteria resist phagocytosis and can persist intracellularly within macrophages for prolonged periods (24).

First significant hints of an influence of *P. acnes* on acne were obtained from *in vivo* studies on the injection of highly concentrated viable propionibacteria into sterile cysts of Steatocystoma multiplex patients; this induced prominent inflammation (25). Intradermal application of dead *P. acnes* cells as well as the injection of viable *Staphylococcus epidermidis* cells were unable to reproduce this effect.

Furthermore, the first use of antibiotics in acne and the clear-cut clinical improvement of acne seen with those agents that reduce *P. acnes*, such as tetracyclines, macrolides, sulfonamides and clindamycin, strengthen the hypothesis that *P. acnes* plays an important role. Likewise the emergence of antibiotic-resistant strains and a concomitant clinical failure further solidifies the importance of *P. acnes* in acne (26). There is evidence that *P. acnes* is involved in invoking an inflammatory response. Although the antigens of *P. acnes* have not been characterized in detail yet, an increased cellular as well as humoral immunity to *P. acnes* could be detected in patients with severe acne. The production of antibodies correlated with the severity of the disease (27, 28). The initial infiltrate into the lesion is lymphocytic, with later progression to a general infiltrate of mixed cell types. CD4+ cells are predominant. CD8+ occur occasionally perivascularly and periductally, and CD1+ cells are present in low numbers (29). Langerhans' cells expressing human leucocyte antigen (HLA)-DR have been observed in close association with perilesional CD4+ T-cells and HLA-DR was shown to be upregulated in the periductal and perivascular infiltrates of acne lesions. Basal keratinocyte expression of HLA-DR has been demonstrated and is indicative of a specific immune response (29).

It is possible that the microorganism and/or its products interact with keratinocytes and sebocytes which then produce cytokines. Cytokines attract lymphocytes non-specifically. Preliminary data suggest that both T-helper 1 (Th1) and Th2 cells play a role in the inflammatory events. Recently it could be demonstrated that *P. acnes* has mitogenic activity. Therefore two mechanisms of lymphocyte activation by *P. acnes* cells are proposed, antigen and mitogen driven (30).

Bacteria and antibiotic resistance

P. acnes is the target of antimicrobial treatments in acne. In 1976, no antibiotic resistance could be detected in *P. acnes* strains from acne patients (31). It was in 1979, when Crawford et al. (32) first described antibiotic resistance of *P. acnes* towards erythromycin and clindamycin in 20% accompanied by therapeutic failure, followed by Eady et al. (26) who reported on resistance to orally applied erythromycin associated with therapeutical failure. The resistance rate increased from 20% in 1978 to 68% in 1996. According to a recent investigation, Mediterranean countries had the highest prevalence of resistance to erythromycin and clindamycin: Spain 91% and 92.4%, respectively; Greece for both erythromycin and clindamycin 75.3%; Italy for both antibiotics 59.5%. A lower resistance rate was detected in northern countries, where it ranged for both substances between 41.5 and 51.4% (33).

Resistance towards minocycline is only 1% (34). Recently, Dreno et al. (35) described a prevalence of bacterial resistance to erythromycin of 95% for *S. epidermidis* strains, 52% for *P. acnes* strains that were colonizing patients with predominantly inflammatory lesions, and 42% of *P. acnes* strains from patients without any previous application of erythromycin. In

Table I. *Diseases (infections/inflammatory conditions) with which* Propionibacterium acnes *has been associated*

Skin manifestation	Ocular manifestation	Others
Acne vulgaris	Acute endophthalmitis	Endocarditis
Periorbital cellulitis	Chronic endophthalmitis	Cerebral absesses
Primary purulent folliculitis	Conjuctivitis	Arthritis
Abscesses	Blepharitis	Osteomyelitis
Kawasaki disease	Keratitis	Meningitis
Sarcoidosis	Canaliculitis, dacryocystitis	Various abscesses (incl. dental)
SAPHO syndrome (acne, palmoplantar pustulosis, osteitis hyperostosis		Dental caries, periodontal disease and gingivitis

Modified from ref. 23

their 10-year surveillance study, Coates et al. (36) showed an increase in antibiotic resistance from 34.5% in 1991 to 64% in 1997. The prevalence then dropped to 50.5% in 1999 and rose again to 55.5% in 2000. Resistance to erythromycin was the most common and most of the strains were cross-resistant to clindamycin. Most patients were colonized with resistant bacterial strains at various sites, including the nares, the latter being difficult to eradicate.

Acne lesions are dominantly colonized by *P. acnes* and *S. epidermidis*. Nishijima et al. (37) isolated *S. epidermidis* and *P. acnes* simultaneously from half of the acne lesions. The application of antimicrobials has to take into consideration that acne lesions are inhabited by both. Therefore, careless administration of antibiotics increases the resistance of several bacteria (34). Nishijima et al. (37) detected antibiotic resistance for more than 30% of *S. epidermidis* strains resistant to erythromycin, roxithromycin, clindamycin. These antibiotics cannot therefore be recommended for long-term (more than 3 months) acne therapy. The ability of *S. epidermidis* to transfer resistance via plasmids to the more pathogenic *S. aureus* has already been observed and taken into consideration (37, 38).

But not only *P. acnes* and *S. epidermidis* develop antibiotic resistance due to acne treatment, and resistance is not only spread among bacteria but resistant bacteria are also spread among patients and their close contacts. Mills et al. (39) have observed that topical treatment with erythromycin can result in a higher carriage rate and dissemination of erythromycin-resistant *S. aureus* from the nares. They observed an increase in the prevalence of erythromycin-resistant coagulase-negative staphylococci from 37% to 88% over the 12-week course of treatment which did not alter in the regression phase, meaning that antibiotic resistance can persist for a considerable time. There is evidence that duration of the treatment period (>6 weeks) has an influence on the persistence of resistant strains. The same study showed that a dissemination of resistant organisms occurred to untreated areas. This implies the transfer of resistant strains to close contacts which has been reported previously (40).

Whereas staphylococci acquire antibiotic resistance very rapidly via plasmids, *P. acnes* develops resistance to tetracyclines, erythromycin and clindamycin over a long period of time via mutational change which is transferred vertically. According to recent results, *P. acnes* antibiotic resistance is commonly associated with mutations in 16S and 23S mRNA, which are present in bacterial isolates from Japan, Australia, U.S.A., and Europe (41). There are additional mechanisms involved which have not yet been characterized (41). The same group found high-level resistant *P. acnes* strains to minocycline in the U.S.A. which is of concern because the resistance may not remain confined to the U.S.A.

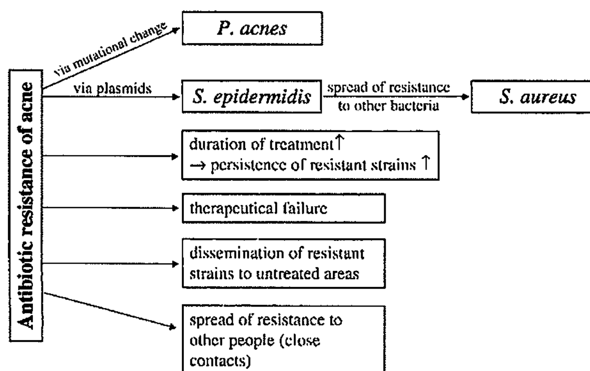

Fig. 2. Consequences of antibiotic resistance in acne. (26, 34, 37–41)

Ross et al. have also discovered a transposon-based macrolide-lincosamide-streptogramin B resistance determinant in cutaneous propionibacteria from six European cities (42). The transposons came from corynebacteria, but the mechanism by which it is transferred into propionibacteria has not been elucidated. The determinant confers a higher degree of resistance than the known 23S rRNA mutations to several macrolide-lincosamide-streptogramin B antibiotics, especially telithromycin and clindamycin, the latter being extensively used in the topical treatment of acne (42).

Further factors

Although early reports and several individual observations indicate that dietary factors, especially chocolate and meat, have an influence on the exacerbation of acne, the association could not be proven. However, there is a significant linear dose-dependent relationship between both acne prevalence and severity and the number of cigarettes smoked daily (43). Cordain et al. (44) reported a very low prevalence of acne in two non-Western societies and observed that the absence of acne is correlated with their diets, which in contrast to a Western diet is significantly less glycaemic. The authors suggest that a diet-induced hyperinsulinaemia elicits endocrine responses that may affect the development of acne through mediators such as androgens, insulin-like growth factor, insulin like growth factor binding protein 3, and retinoid signalling pathways. Further studies on these interactions may be useful in the treatment of acne in Western populations.

DIAGNOSTIC PROCEDURES

The first examination should include inquiries on familiar occurrences, on cosmetics used, menstrual cycle irregularity indicating hyperandrogenism, drugs like vitamin B, lithium, phenytoin, which aggravate acne, further skin diseases and non-skin diseases, particularly endocrinologic and metabolic disturbances. Facing the

growing significance of antibiotic resistance, facial skin swabs should be investigated for *P. acnes* and staphylococci including resistance analysis (reviewed in 45), which has not been introduced as a routine test due to a lack of appropriate facilities for *P. acnes* cultivation in dermatological practice. Female patients with clinical signs for hyperandrogenaemia should be investigated for the concentrations of testosterone, prolactin, sex hormone binding globulin (SHBG), DHEAS, cortisol, estradiol. Patients with a severe course of the disease should additionally be examined for erythrocyte sedimentation rate, C-reactive protein, differential blood count, immunoglobulins, zink and α1-antitrypsin (45).

TREATMENT OF ACNE

The aims of treatments are to improve the disfiguring, the psychological distress, and to prevent scarring. According to the aetiopathological factors in acne, the therapy consists of anticomedogenic, anti-inflammatory and antimicrobial substances. Drugs that reduce comedones are the topical retinoids (tretinoin, tazarotene, adapalene and retinylaldehyde) and oral isotretinoin.

Retinoids

Retinoids do have an effect on cell proliferation and differentiation, are sebostatic and keratolytic but also have anti-inflammatory properties. Isotretinoin given orally induces sebaceous gland size reduction to up to 90% by decreasing proliferation of basal sebocytes, suppressing sebum production and inhibiting sebocyte differentiation *in vivo*. Its unique activity on sebaceous glands is still not clearly understood. However, in 2000, Tsukada et al. (46) reported the mechanism by which 13-*cis* retinoic acid is sebocyte-specific. They provided evidence that the so-called specific antisebotropic activity of 13-*cis* retinoic acid is likely to be mediated by a selective rapid and marked intracellular isomerization of 13-*cis* retinoic acid to all-*trans* retinoic acid in human sebocytes with subsequent binding of all-*trans* retinoic acid to and activation of retinoic acid receptors, supporting a pro-drug/drug relation between the two compounds (46).

It has also been suggested from investigations of biopsy material that retinoids have a direct pharmacological action on neutrophils. Topical tretinoin has been shown to have an inhibitory effect on the release of lysosomal enzymes from polymorphonuclear leucocytes, enzymes playing a key role in follicular wall damage, thereby releasing inflammatory mediators into the dermis (47). Tretinoin and adapalene were shown to inhibit human polymorphonuclear neutrophil lipoxygenase activity (48), the latter being essential for the oxidative metabolism of arachidonic acid, a pathway in the production of inflammatory mediators. The systemic retinoid, isotretinoin, is used for severe cystic and scarring acne, acne that is resistant to oral antibiotics and acne that quickly relapses after antibiotic therapy. Serious side effects like teratogenicity, myalgias, arthralgias have to be considered, and the patient has to sign informed consent. Recently, several cases of psychological depression and even suicide have been reported among patients treated with isotretinoin. However, the causal relationship between isotretinoin therapy and depression has not been clearly established and needs further study (49, 50).

Micronized isotretinoin caused fewer and less intense mucocutaneous adverse events than standard isotretinoin, which may improve patients' quality of life (51).

Similar to other topically applied retinoids, adapalene shows antiproliferative and prodifferentiating effects on keratinocytes but less potential for irritation and a high lipophilicity (52). As to antimicrobial effects, adapalene has no direct influence on *P. acnes* itself. There is evidence that it reduces the inflammatory response to bacterial antigens and mediators. Therefore, it does not support the development of antibiotic resistance in *P. acnes*.

Hormonal treatments

Hormonal treatments of acne consist of the ovarian suppression of androgen production by oral contraceptives, androgen receptor blockers: cyproterone acetate and spironolactone, adrenal suppression of androgen production by corticosteroids and inhibitors of 5-α-reductase. Hormones are not the first choice treatment, only for women with mild acne or signs of hyperandrogenism who ask for contraception. Pills with an extremely low androgenic progestin concentration, or – even more efficient – with a low dose of 2 mg of cyproterone acetate should be used.

Antimicrobial substances

Antibiotics reduce the bacterial colonization of the deeper parts of the follicle. *P. acnes* is sensitive to a wide range of antimicrobials *in vitro*, but only lipophilic drugs penetrate the microcomedo and are bacteriologically and clinically efficient in patients with acne, which is the case for tetracycline, doxycycline and minocycline. These antibiotics have additional anti-inflammatory capacities, particularly minocycline. For topical application erythromycin, gentamycin, tetracycline as well as clindamycin are used. Topical erythromycin is not only well accepted, it also has anti-inflammatory properties, suppresses the chemotaxis of inflammatory cells and decreases pro-inflammatory free fatty acids in sebum indirectly by down-regulating either the *P. acnes* metabolism and/or extracellular lipase production, the latter being also affected by tetracyclines (53–56). However, it is highly advisable not to use topical antibiotics alone because of the risk of the development of antibiotic resistance (57) but instead to combine it

with other topical substances, for example, benzoyl peroxide (BPO), azelaic acid, tazarotene, tretinoin, adapalene, which also have either direct or indirect antimicrobial activity (52, 58). Out of the retinoids retinoic acid, retinol and retinaldehyde, which are currently used in many formulations, retinaldehyde has been shown to have significant direct antibacterial activities upon topical use without the emergence of resistant strains (59). The combination of erythromycin or clindamycin and BPO topically is recommended because the number of aerobic bacteria is reduced without any change in the resistance towards erythromycin or other antibiotics (31, 57, 60). Cunliffe (34) recommended the combination of BPO + Adapalene + topical antibiotic to reduce the resistance rate. Pfannschmidt et al. (61) demonstrated that the combination of topical tretinoin and erythromycin was more effective than either agent used alone. In patients with comedones and papulopustular lesions a therapy with a topical retinoid and either a topical or systemic antibiotic is the preferred approach (62).

New therapeutical strategies

Because most retinoids do not have a direct effect on *P. acnes*, this group of substances is not able to rectify the four major components of acne pathogenesis. Monotherapy, therefore, is mostly not efficient. Nor should topical antibiotics, which apart from antimicrobial activity also have anti-inflammatory properties, be used as monotherapy because of the development of antibiotic resistance. Future strategies for the use of antimicrobial therapy in patients with acne should consist of minimization of antibiotic use, and the combination of antibiotics with other substances. Oral antibiotic treatment should be stopped once control is achieved. Maintenance therapy should consist of either topical retinoids and benzoyl peroxide or benzoyl peroxide and antibiotic (63). And whichever strategy is used, it should be started as early as possible (52).

As to the development of new substances and their introduction into medical practice, a topical antiandrogenic treatment would be an interesting approach. So far it has been disappointing probably because *P. acnes* is able to metabolize androgens applied to the skin, therefore, the development of combinations of antiandrogen and antimicrobial agents should be considered. A relatively new antibiotic is lymecycline, which is as effective as minocycline but has fewer side effects (64).

Zouboulis et al. (65) demonstrated a 70% reduction in inflammatory lesions after 3 months of therapy with a new anti-inflammatory agent that specifically blocks the formation of leukotriene B4. In parallel, these patients showed a reduction in total lipids, hydroperoxides and free fatty acids in sebum. Recently, the efficacy of topical 5-aminolaevulinic acid photodynamic therapy and selectivity of photosensitizer accumulation has been demonstrated in the treatment of both truncal and facial acne (66).

Vaccination

According to a single publication, an oral antigen treatment with heat inactivated lyophilized acne bacteria had been performed in the early 1970s and could be shown to have improved acne in 80% of cases (67). Since there is evidence that *P. acnes* is associated with inflammation in acne and that the microorganism rises a humoral as well as a cellular immune response the idea of a *P. acnes*-based vaccination is tempting. However, the multifactorial character of acne has to be considered.

CONCLUSION

The increasing importance of appearance and the influence of disfiguring on psychologic health confirm the significance of the disease "acne". Although during the past 50 years dramatic progress has been made concerning the development of treatment modalities, the pathomechanism is still not fully understood. Molecular biology combined with molecular immunology and pharmaceutical research is necessary to clarify these issues. The current recommendations for the use of antibiotics in acne is summarized in Table II.

Table II. *Recommendations for the use of antibiotics in acne therapy*

1. Prescribe antibiotics only if necessary.
2. Treatment for as short a time as possible accepting that 6 months is the minimum for oral and 3 months for topical therapy.
3. When further treatment is necessary, re-use the same antibiotic (unless it loses efficacy).
4. Apply benzoyl peroxide (BPO) for a minimum of 5–7 days between antibiotic courses to eliminate resistant organisms from the skin (which unfortunately may persist in the nares).
5. Avoid changes of therapeutic protocols unless necessary.
6. Avoid concomitant use of oral and topical therapy with chemically dissimilar antibiotics.
7. Inform and educate the patient concerning compliance and treatment strategies.
8. Stop antibiotic treatment once control is achieved.
9. Institute maintenance therapy consisting of either topical retinoids and BPO or BPO and topical antibiotic.

Modified from ref. 68

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REFERENCES

1. Gupta MA, Gupta AK. Depression and suicidal ideation in dermatology patients with acne, alopecia areata, atopic dermatitis and psoriasis. Br J Dermatol 1998; 139: 846–850.
2. Cunliffe WJ. Natural history of acne. In: Cunliffe WJ, ed. Acne. London: Martin Dunitz, 1989: 2–10.
3. Gollnick H, Zouboulis ChC, Akamatsu H, Kurokawa I , Schulte A. Pathogenesis and pathogenesis related treatment of acne. J Dermatol 1991; 18: 489–499.
4. Harris HH, Downing DT, Stewart ME, Strauss JS. Sustainable rates of sebum secretion in acne patients and matched normal control subjects. J Am Acad Dermatol 1983; 8: 200–203.
5. Bläuer M, Vaalasti A, Pauli SL, Ylikomi T, Joensuu T, Tuohimaa P. Location of androgen receptor in human skin. J Invest Dermatol 1991; 97: 264–268.
6. Choudhry R, Hodgins MB, Van der Kwast TH, Brinkmann AO, Boersma WJ. Localisation of androgen receptors in human skin by immunohistochemistry: implications for the hormonal regulation of hair growth, sebaceous glands and sweat glands. J Endocrinol 1992; 133: 467–475.
7. Thigpen AE, Silver RI, Guileyardo JM, Casey ML, McConnell JD, Russell DW. Tissue distribution and ontogeny of steroid 5α-reductase iso-enzyme expression. J Clin Invest 1993; 92: 903–910.
8. Luu-The V, Sugimoto Y, Puy L, Labrie Y, Lopez-Solache I, Singh M, et al. Characterization, expression, and immunohistochemical localization of 5-α-reductase in human skin. J Invest Dermatol 1994; 102: 221–226.
9. Thiboutot D, Harris G, Iles V, Cimis G, Gilliland K, Hagari S. Activity of the type I 5 α-reductase exhibits regional differences in isolated sebaceous glands and whole skin. J Invest Dermatol 1995; 105: 209–214.
10. Chen WC, Zouboulis ChC, Orfanos CE. The 5 α-reductase system and its inhibitors: recent development and its perspective in treating androgen-dependent skin disorders. Dermatology 1996; 193: 177–184.
11. Zouboulis CC, Seltmann H, Hiroi N, Chen WC, Young M, Oeff M, et al. Corticotropin-releasing hormone: an autocrine hormone that promotes lipogenesis in human sebocytes. www.pnas.org/cgi/doi/10.1073
12. Weeks JG, McCarty L, Black T, Fulton JE. The inability of bacterial lipase inhibitor to control acne vulgaris. J Invest Dermatol 1977; 69: 236–243.
13. Downing DT, Stewart ME, Wertz PW, Strauss JS. Essential fatty acids and acne. J Am Acad Dermatol 1986; 14: 221–225.
14. Zouboulis C. Update on sebaceous gland physiology: induction of inflammation and its clinical implications. JEADV 2001; 15 Suppl. 2: 102.
15. Guy R, Kealey T. Modelling in acne. Dermatology 1998; 196: 32–37.
16. Ingham E, Eady EA, Goodwin CE, Cove JH, Cunliffe WJ. Pro-inflammatory levels of interleukin-1α-like bioactivity are present in the majority of open comedones in acne vulgaris. J Invest Dermatol 1992; 98: 895–901.
17. Eller MS, Yaar M, Ostrom K, Harkness DD, Gilchrest BA. A role for interleukin-1 in epidermal differentiation: regulation by expression of functional versus decoy receptors. J Cell Sci 1995; 108: 2741.
18. Akamatsu H, Horio T. The possible role of reactive oxygen species generated by neutrophils in mediating acne inflammation. Dermatology 1998; 196: 82–85.
19. Marples RR, McGinley KJ. Corynebacterium acnes and other anaerobic diphtheroids from human skin. J Med Microbiol 1974; 7: 349–357.
20. Holland KT. Microbiology of acne. In: Cunliffe WJ, ed. Acne. London: Martin Dunitz, 1989: 178–210.
21. Leyden JJ, McGinley KJ, Mills OH, Kligman AM. Age related changes in the resident bacterial flora of the human face. J Invest Dermatol 1975; 65: 379–381.
22. McGinley KJ. Regional variations in the density of cutaneous propionibacteria: correlation of Propionibacterium acnes populations with sebaceous secretions. J Clin Microbiol 1980; 12: 672–675.
23. Eady EA, Ingham E. Propionibacterium acnes – friend or foe. Rev Med Microbiol 1994; 5: 163–173.
24. Webster GF, Leyden JJ, Musson RA, Douglas SD. Susceptibility of Propionibacterium acnes to killing and degradation by human neutrophils and monocytes in vitro. Infect Immun 1985; 49: 116–121.
25. Kirschbaum JD, Kligman AM. The pathogenic role of Corynebacterium acnes in acne vulgaris. Arch Dermatol 1963; 88: 832–833.
26. Eady EA, Cove JH, Holland KT, Cunliffe WJ. Erythromycin-resistant propionibacteria in antibiotic-treated acne patients. Association with therapeutic failure. Br J Dermatol 1989; 121: 51–57.
27. Holland KT, Holland DB, Cunliffe WJ, Cutcliffe AG. Detection of Propionibacterium acnes polypeptides which have stimulated an immune response in acne patients but not in normal individuals. Exp Dermatol 1993; 2: 12–16.
28. Ashbee HR, Muir SR, Cunliffe WJ, Ingham E. IgG subclasses specific to Staphylococcus epidermidis and Propionibacterium acnes in patients with acne vulgaris. Br J Dermatol 1997; 136: 730–733.
29. Layton AM, Morris C, Cunliffe WJ, Ingham E. Immunohistochemical investigation of evolving inflammation in lesions of acne vulgaris. Exp Dermatol 1998; 7: 191–197.
30. Jappe U, Ingham E, Henwood J, Holland KT. Propionibacterium acnes and inflammation in acne. P. acnes has T-cell-mitogenic activity. Br J Dermatol 2002; 146: 202–209.
31. Leyden JJ. Antibiotic resistant acne. Cutis 1976; 17: 593–596.
32. Crawford WW, Crawford IP, Stoughton RB, Cornell RC. Laboratory induction and clinical occurrence of combined clindamycin and erythromycin resistance in Corynebacterium acnes. J Invest Dermatol 1979; 72: 187–190.
33. Ross JI, Carnegie E, Snelling AM, Coates P, Cove JH, Eady EA, et al. Prevalence of antibiotic resistant propionibacteria on the skin of acne patients from six European countries. JEADV 2001; 15 Suppl. 2: 135.
34. Cunliffe WJ. Propionibacterium acnes resistance and its clinical relevance. J Dermatol Treatment 1995; 6 Suppl. 1: S3–S4.
35. Dreno B, Reynaud A, Moyse D, Habert H, Richet H. Erythromycin-resistance of cutaneous bacterial flora in acne. Eur J Dermatol 2001; 11: 549–553.
36. Coates P, Vyakrnam S, Eady EA, Jones CE, Cove JH, Cunliffe WJ. Prevalence of antibiotic-resistant propionibacteria on the skin of acne patients: 10-year surveillance

data and snapshot distribution study. Br J Dermatol 2002; 146: 840–848.
37. Nishijima S, Kurokawa I, Katoh N, Watanabe K. The bacteriology of acne vulgaris and antimicrobial susceptibility of Propionibacterium acnes and Staphylococcus epidermidis isolated from acne lesions. J Dermatol 2000; 27: 318–323.
38. Jaffe HW, Sweeney HM, Nathan C, Weinstein RA, Kabins SA, Cohen S. Identity and interspecific transfer of gentamicin-resistance plasmids in Staphylococcus aureus and Staphylococcus epidermidis. J Infect Dis 1980; 141: 738–747.
39. Mills O, Thornsberry C, Cardin CW, Smiles KA, Leyden JJ. Bacterial resistance and therapeutic outcome following three month of topical acne therapy with 2% erythromycin gel versus its vehicle. Acta Derm Venereol 2002; 82: 260–265.
40. Miller YW, Eady EA, Lacey RW, Cove JH, Joanes DN, Cunliffe WJ. Sequential antibiotic therapy for acne promotes the carriage of resistant staphylococci on the skin of contacts. J Antimicrob Chemother 1996; 38: 829–837.
41. Ross JI, Snelling AM, Eady EA, Cove JH, Cunliffe WJ, Leyden JJ, et al. Phenotypic and genotypic characterisation of antibiotic-resistant Propionibacterium acnes isolated from acne patients attending dermatology clinics in Europe, the USA, Japan and Australia. Br J Dermatol 2001; 144: 339–346.
42. Ross JI, Eady EA, Carnegie E, Cove JH. Detection of transposons Tn5432-mediated macrolide-lincosamide-streptogramin B (MLSB) resistance in cutaneous propionibacteria from six European cities. J Antimicrob Chemother 2002; 49: 165–170.
43. Schaefer T, Nienhaus A, Vieluf D, Berger J, Ring J. Epidemiology of acne in the general population: the risk of smoking. Br J Dermatol 2001; 145: 100–104.
44. Cordain L, Lindeberg S, Hurtado M, Hill K, Eaton SB, Brand-Miller J. Acne vulgaris. A disease of Western civilization. Arch Dermatol 2002; 138: 1584–1590.
45. Gollnick HPM. Guidelines "Acne and acne variants". Hautarzt 2002; 53: 322–327.
46. Tsukada M, Schröder M, Roos TC, Chandraratna RAS, Reichert U, Merk HF, et al. 13-cis retinoic acid exerts its specific activity on human sebocytes through selective intracellular isomerization to all-trans retinoic acid and binding to retinoic acid receptors. J Invest Dermatol 2000; 115: 321–327.
47. Camisa C, Eisenstat B, Ragaz A, Weissmann G. The effects of retinoids on neutrophil functions in vitro. J Am Acad Dermatol 1982; 6: 620–629.
48. Shroot B. Pharmacodynamics and pharmacokinetics of topical adapalene. J Am Acad Dermatol 1998; 39: S17–S24.
49. Jacobs DG, Deutsch NL, Brewer M. Suicide, depression, and isotretinoin: is there a causal link? J Am Acad Dermatol 2001; 45 Suppl. 1: 68–75.
50. Ng CH, Schweitzer I. The association between depression and isotretinoin use in acne. Aust NZ J Psychiatry 2003; 37: 78–84.
51. Strauss JS, Leyden JJ, Lucky AW, Lookingbill DP, Drake LA, Hanifin JM, et al. Safety of a new micronized formulation of isotretinoin in patients with severe recalcitrant nodular acne: a randomized trial comparing micronized isotretinoin with standard isotretinoin. J Am Acad Dermatol 2001; 45: 196–207.
52. Shalita A. The integral role of topical and oral retinoids in the early treatment of acne. JEADV 2001; 15 Suppl. 3: 43–49.
53. Plewig G, Schöpf E. Anti-inflammatory effects of antimicrobial agents: an in-vivo study. J Invest Dermatol 1975; 65: 532–536.
54. Esterly NB, Furey NL, Flanagan LE. The effect of antimicrobial agents on leukocyte chemotaxis. J Invest Dermatol 1978; 70: 51–55.
55. Fulton JE, Pablo G. Topical antibacterial therapy for acne. Study of the family of erythromycins. Arch Dermatol 1974; 110: 83–86.
56. Webster GF, McGinley KJ, Leyden JJ. Inhibition of lipase production in Propionibacterium acnes by subminimal-inhibitory concentrations of tetracycline and erythromycin. Br J Dermatol 1981; 104: 453–457.
57. Leyden J, Levy S. The development of antibiotic resistance in Propionibacterium acnes. Cutis 2001; 67: 21–24.
58. Holland KT, Bojar RA. Antimicrobial effects of azelaic acid. J Dermatol Treat 1993; 4 Suppl. 1: S8–11.
59. Pechere M, Germanier L, Siegenthaler G, Pechere JC, Saurat JH. The antibacterial activity of topical retinoids: the case of retinaldehyde. Dermatology 2002; 205: 153–158.
60. Ellis CN, Leyden J, Katz HI, Goldfarb MT, Hickman J, Jones TM et al. Therapeutic studies with a new combination benzoyl peroxide/clindamycin topical gel in acne vulgaris. Cutis 2001; 67 Suppl. 2: 13–20.
61. Pfannschmidt N, Bauer R, Kreysel HW. Combined topical treatment of acne with erythromycin and tretinoin. Z Hautkr 1998; 63: 366–368.
62. Leyden JJ. Topical treatment for acne vulgaris. N Engl J Med 1997; 336: 1156–1162.
63. Leyden JJ. Current issues in antimicrobial therapy for the treatment of acne. JEADV 2001; 15 Suppl. 3: 51–55.
64. Grosshans E, Belaich S, Meynadier J, Alirezai M, Thomas L. A comparison of the efficacy and safety of lymecycline and minocycline in patients with moderately severe acne vulgaris. Eur J Dermatol 1998; 8: 161–168.
65. Zouboulis CC, Nestoris S, Adler YD, Picardo M, Camera E, Orth M, et al. Treatment of inflammatory acne with an oral lipoxygenase inhibitor. J Invest Dermatol 2001; 117: 547.
66. Ibbotson SH. Topical 5-aminolaevulinic acid photodynamic therapy for the treatment of skin conditions other than non-melanoma skin cancer. Br J Dermatol 2002; 146: 178–188.
67. Niedre W. Oral antigen treatment (oral vaccination) in acne diseases. Z Allgemeinmed 1975; 20: 227–228.
68. Eady EA. Bacterial resistance in acne. Dermatology 1998; 196: 59–66.

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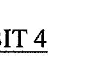

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Assessment of Etiologic Agents in Acne Pathogenesis

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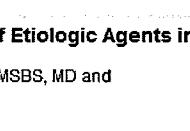

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Acne is a chronic inflammatory disease of the pilose-baceous units. Traditional etiologic factors include increased sebum production, ductal hyperkeratosis, abnormality of the microbial flora within the pilose-baceous unit, and mediators of inflammation. Recent developments do not refute these familial elements, but rather refine particular aspects. Interleukin-1α influences hypercornification of the infundibulum as well as the inflammatory response by inducing the production of vascular endothelial growth factor in dermal papilla cells and follicular keratinocytes of the pilosebaceous unit. New retinoids have been developed based on controlling cellular proliferation and differentiation in the pilosebaceous unit by their action on nuclear receptors of cells. Dermal inflammation is not due to presence of bacteria, but from biologically active mediators produced by Propionibacterium acnes. The environment within the pilosebaceous unit is probably more important than the absolute number of P. acnes *organisms. Indeed, the major role of the sebaceous gland appears to be supplying* P. acnes *needed nutrients. Moreover, the microbiologic principle ofbiofilms appears to be applicable to P. acnes in acne.*

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EXHIBIT 5

AEROBIOLOGIA
9 (1993), 69 - 74

Population study of atmospheric bacteria at the Fengtai district of Beijing on two representative days

TONG YONGYI, CHE FENGXIANG, XU XIUZHI, CHEN MIELENG, YE BINYAN, JIANG LI

SUMMARY. We collected bacteria from the atmosphere (5-6 m above ground level) with an Andersen microbial sampler at Fengtai District, Beijing, in the spring and autumn of 1989. The 195 strains of bacteria collected and isolated were identified according to their morphological, biochemical and physiological characteristics. The results showed that there were 11 bacterial genera in the atmosphere of the town, the most abundant being Staphylococcus *and* Bacillus. *The order of prevailing genera of the two seasons is similar. In morphology, gram positive (G^+) bacteria predominate among atmospheric bacteria (G^+ cocci, G^+ rods and G^+ endospore-forming rods). It seems that the constitution of atmospheric bacteria is influenced not only by the source strength of the various bacteria in the environment, but also by their somewhat resistant mechanisms such as protective pigments and spore structure. From our experience, in order to avoid the possible data loss from selecting and identifying some «representative» colonies of samples, all strains isolated should be identified when studying ecological distribution of microbes in the atmosphere.*

Key words: atmospheric bacteria, identification, population distribution.

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INTRODUCTION

Background investigation of microbes in the atmosphere is very important in order to understand the role of these microbes in the ecosystem, and to control air pollution caused by hazardous microbes. Although there are some reports on the concentration and size distribution of particles containing various microbes in the atmosphere (Bovallius *et al.*, 1978; Jones *et al.*, 1983; Lee *et al.*, 1973; Wright *et al.*, 1969; Hu Qingxuan *et al.*, 1990), the data on population distribution of atmospheric microbes are limited (Che Fengxiang *et al.*, 1990; Mancinelli *et al.*, 1978; Xi Suming *et al.*, 1988). We collected bacterial samples from the atmosphere of Fengtai district, Beijing, during two days in the spring and autumn of 1989, and identified the 195 strains of bacteria according to their morphological, biochemical and physiological characteristics.

MATERIALS AND METHODS

Sampling location: We collected airborne bacteria on top (about 5-6 m above ground level) of a hall at 23 (A) Qilizhuang Road, Fengtai. Fengtai is a satellite town located south-west to the suburb area of Beijing with dense population and relatively little industry activity. Agrarian area surrounds the town.

Sampling time: Airborne bacteria were sampled at 9:30 am on April 16th and October 4th, 1989, which represent typical spring and autumn days in Beijing, respectively. The two sampling events took place on clear days with temperature around 16-23 °C and moderate wind (3-6 m/s).

Device and sampling method: An Andersen microbial sampler was used to collect airborne bacteria (Andersen, 1958). Collection medium consisted of peptone (10 g), beef extract (3-5 g), NaCl (5 g), agar (18-20 g) in 1 liter of distilled water (PH: 7.4-7.6). The sampler was sterilized with epoxyethane before use. To prevent the possible contamination and contribution from operator, the operator wore clean garments and a gauze mask then sterilized his hands with 70% ethanol. After having sampled for 1 min at a flowrate of 28.3 l/min, plates were removed from the sampler in sterile manner and sent to an incubator.

Isolation and identification of bacteria: After incubation for 48 h at 30 °C, all visible colonies were picked out and streaked onto new plates for further purification. After the purity of an isolate was confirmed by colony appearances and Gram stain features, it was identified according to its morphology, biochemical and physiological tests with reference to Bergey's Manual of Systematic Bacteriology (Holt *et al.*, 1984, 1986; Buchanan *et al.*, 1978) and Methods for Identification of General Bacteria (The Group of Classification of Bacteria, Institute of Microbiology, 1978). The tests used for identification in this study are listed in Tab. I. When more than one kind of bacteria was isolated from a single original colony, all the strains were identified.

Table I. Tests adopted in this study for Bacterial Identification.

Growth characteristics	on nutrient agar plate on nutrient agar slant in nutrient broth
Stain characteristics	Gram stain Flagellus stain Spore stain β-hydroxybutyric acid stain
Biochemical and physiological tests	Arginine dihydrolase reaction Atmospheric oxygen requirement Catalase reaction Cellulase activity Coagulase test (rabbit plasma) Gas/acid production from arabinose, glucose, mannose, mannitol, selicine, trehalose, xylose) Gelatin liquefaction Growth in nutrient broth at pH 5.7 H_2S production Hugh and leifson test (O-F test) Maximum NaCl tolerance tests Methyl red test (MR test) Motility test Nitrate reduction nitrogen fixation test Oxidase reaction Survival heating in skin milk at 63°C for 30 min or at 72°C for 15 min Starch hydrolysis Utilization of propionate Voges-Proskauer test (V-P test)

RESULTS

1. *Population distribution of atmospheric bacteria:* In the spring sample the 81 strains of bacteria were distributed among 7 genera, while in the autumn sample 114 strains were distributed among 11 different genera. *Staphylococcus* and *Bacillus* are the prevailing genera in both seasons. In spring, the relative order of dominant bacterial genera in importance was as follows: *Staphylococcus, Bacillus, Listeria, Micrococcus, Corynebacterium, Brevibacterium.* In autumn, the order was *Staphylococcus, Bacillus, Listeria, Corynebacterium, Micrococcus* (Tab. II).

Table II. Population distribution of atmospheric bacteria at Fengtai in 1989.

Genus	Spring (7 genera)		Autumn (11 genera)	
	Number of strains	%	Number of strains	%
Staphylococcus	26	32.1	33	28.9
Micrococcus	8	9.9	5	4.4
Planococcus	0	0.0	1	0.9
Paracoccus	0	0.0	1	0.9
Corynebacterium	8	9.9	18	15.8
Brevibacterium	6	7.4	4	3.5
Listeria	11	13.6	19	16.7
Kurthia	0	0.0	2	1.8
Acinebacter	1	1.2	1	0.9
Bacillus	21	25.9	29	25.4
Other	0	0.0	1	0.9
Total	81	100.0	114	100.0

Two prevailing genera, *Staphylococcus* and *Bacillus,* were further identified to the species level. The results are presented in Tab. III and Tab. IV. *Staphylococcus aureus,* which are conditional pathogenic bacteria to human beings, are differentiated from other species of *Staphylococcus* according to plasma coagulase and mannitol tests. We can see that *Staphylococcus aureus* composes a certain fraction of *Staphylococcus* population in both seasons, and that the autumn fraction in somewhat higher than the spring one. There are 9 different species of *Bacillus* (Tab. IV). Among the population of *Bacillus, Bacillus firmus* in the most frequent species in spring and *Bacillus cereus* is frequent in autumn.

2. *Morphological features of atmospheric bacteria:* Gram stain features of atmospheric bacteria are shown in Tab. V. Most of the atmospheric bacteria are gram positive (G^+) bacteria (G^+ cocci, G^+ rods and G^+ endospore-forming rods), whereas gram negative bacteria compose only a very small fraction of atmospheric bacteria. The result is similar to that of Che Fengxiang (1990).

3. *Colony colour features of atmospheric bacteria:* The colony colours of several main atmospheric bacteria were analysed and the results are shown in Tab. VI. The bacteria with carotene pigments (yellow or reddish) are the prevailing ones among atmospheric bacteria. This is especially true for two kinds of cocci (*Staphylococcus* and *Micrococcus).* Except for carotene pigments, milky white is another popular colour among G^+ rods (*Corynebacterium, Brevibacterium, Listeria).* Most *Bacillus* form milky white colonies.

4. One positive hole on a plate may carry more than one kind of bacteria on it. Since the number of «positive holes» at each stage of an Andersen sampler is around 20 in autumn sample, multiple growth rarely occurred. In Tab. VII, we can see that although most of impaction groups carry a single kind a bacteria with them, sometimes (in about 18% of cases) a single impaction group carries two or more kind of bacteria.

Table III. The percentage of *Staphylococcus aureus* among *Staphylococcus* population in the atmosphere of Fengtai, Beijing in 1989.

Seasons	*Number of strains of* Staphylococcus	*Number of strains of* S. aureus	*Percentage of* S. aureus *among* Staphylococcus *(%)*
Spring	26	2	7.7
Autumn	33	7	21.7
Total	59	9	15.3

Table IV. Species of *Bacillus* in the atmosphere at Fengtai, Beijing in 1989.

Species of Bacillus	*Spring (6 species)*		*Autumn (7 species)*	
	Number strains	*%*	*Number of strains*	*%*
B. cereus	0	0.0	9	31.0
B. megaterium	1	4.8	7	24.1
B. licheniformis	3	14.3	4	13.8
B. firmus	10	47.6	3	10.3
B. coagulans	0	0.0	3	10.3
B. brevis	2	9.5	0	0.0
B. pumilus	3	14.3	2	6.9
B. anthracoides	0	0.0	1	3.4
B. stearothermophilus	2	9.5	0	0.0
Total	21	100.0	29	100.0

Table V. Morphological features of atmospheric bacteria at Fengtai town in 1989.

Morphological features	*Spring*		*Autumn*	
	Number of strains	*%*	*Number of strains*	*%*
G+ cocci	34	42.0	39	34.2
G+ rods	25	30.9	43	37.7
G+ endospore-forming rods	21	25.9	29	25.4
G− rods	1	1.2	2	1.8
G− cocci	0	0.0	1	0.9
Total	81	100.0	114	100.0

Table VI. Colony colour features of atmospheric bacteria.

Colony Colour	*Staphylococcus*		*Micrococcus*		*Corynebacterium*		*Brevibacterium*		*Listeria*		*Bacillus*	
	No.	*%*	*No.*	*%*	*No.*	*%*	*No.*	*%*	*No.*	*%*	*No.*	*%*
Milk white	3	5.1	0	0.0	15	57.7	4	40.0	8	26.7	33	66.0
Almond yellow	6	10.2	0	0.0	5	19.2	1	10.0	2	6.7	1	2.0
Jasmine yellow		1.7	0	0.0	2	7.8	0	0.0	1	3.3	0	0.0
Wheat straw yellow	5	8.5	1	7.8	0	0.0	0	0.0	0	0.0	0	0.0
Rape flower yellow	6	10.2	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0
Junket yellow	4	6.8	0	0.0	2	7.8	0	0.0	1	3.3	3	6.0
Egg shell yellow	18	30.5	5	38.5	2	7.8	5	50.0	11	36.7	12	24.0
Apricot yellow	16	27.1	6	46.3	0	0.0	0	0.0	7	23.3	1	2.0
Realgar yellow	0	0.0	1	7.8	0	0.0	0	0.0	0	0.0	0	0.0
Total	50	100.0	13	100.0	26	100.0	10	100.0	30	100.0	50	100.0

Table VII. Relationship between the particle size and the kinds of bacteria carried on the particle (Autumn 1989).

Stages of Andersen sampler & particle size collected on each stage (μm)	*Colonies collected on each stage*	*Colony Forming Units 1 Kind bacteria*		*Colony Forming Units 2 kind bacteria*		*Colony Forming Units 3 kind bacteria*	
		Number	*%*	*Number*	*%*	*Number*	*%*
1 (<8.2)	18	16	88.9	1	5.6	1	5.6
2 (5.0-10.4)	13	11	84.6	2	15.4	0	0.0
3 (3.0- 6.0)	15	14	93.3	1	6.7	0	0.0
4 (2.0- 3.5)	6	3	50.0	3	50.0	0	0.0
5 (1.0- 2.0)	20	16	80.0	3	15.0	1	5.0
6 (<1.0)	23	18	78.3	5	21.7	0	0.0
Total	95	78	82.1	15	15.8	2	2.1

DISCUSSION

Seemingly the contents of atmospheric microbes is influenced by the source strength of various microbes originating in the surroundings and their survival in the air. Fengtai is a satellite town which is densely populated and surrounded by agrarian environment. Bare soil, agrarian activity, living and production activities are the sources of local atmospheric bacteria. The abundance of *Staphylococcus* in the atmosphere is easily explainable, since the bacteria are widely distributed on the skin and inside the respiratory tract of people and can enter the atmosphere as aerosols through shedding and coughing. Organic garbage which is rich in *Staphylococcus* is another source of airborne bacteria in atmosphere, mainly by entrainment. *Staphylococcus aureus* composes an important fraction among the populations of *Staphylococcus* in both of our sampling seasons. Dense human population in the town and the animals of a nearby experimental farm may be responsible for the occurrence of the bacteria in the air. Besides, we observed that many *Staphylococcus* had yellow or orange pigments (Tab. VI). These pigments are thought to provide bacteria with resistance to sun radiation. Imshenetsky (1978) collected 6 strains of microbes from the mesopshere (60-80 km), 5 of which had some pigments. This leads us to believe that the protective pigment is a characteristic of atmospheric microbes or a phenomenon of natural selection occurring in the atmosphere. The wide distribution of *Bacillus* in the soil and its resistant spores may be responsible for their abundance in the atmosphere. Gram negative bacteria are abundant on plant leaves and in the soil, but they were rarely collected in this study. This may be due to their weak resistance to air stresses such as dessication, sun radiation and aerosolized chemical pollutants (Tong Yongyi *et al.*, 1990; Xi Suming *et al.*, 1988 and Mancinelli *et al.*, 1978) found that *Micrococcus* was another important bacteria in the atmosphere in addition to *Staphylococcus* and *Bacillus*. Our results show that *Micrococcus* is not as important as the latter. So, geographical and environmental features may cause prevailing atmospheric bacterial pattern to differ from place to place.

The appearance of some conditional pathogens such as *Staphylococcus aureus* and *Bacillus cereus* in the urban atmosphere suggests that it is urgent to take measures to reduce pollution sources and control the quality of our atmosphere.

Since the number of «positive holes» at each stage of Andersen sampler is around 20, we exclude the possibility of multiple impaction on the same hole. So we assume that one bacterial laden particle grows into one colony. Once that all of the strains isolated from a single colony in the autumn sample were identified, it resulted that about 18% of the bacterial laden particles were carrying more than one kind of bacteria on them. Obviously, all strains isolated should be identified in order to avoid the loss of information when the ecological distribution of microbial population in the atmosphere is studied. Of course, in order to identify such numerous isolates, rapid and automatic devices or methods such as VITEK-AMS system are needed. Otherwise it would be difficult to achieve results.

CONCLUSIONS

Natural processes and human activities produce various aerosols, in which not only organic and inoganic substances, but also various microbes are contained. These particles have complex influences on the health of human beings and on natural environments. It is a pity that we have often ignored other aspects while we have concentrated on one aspect only due to the limits imposed by the activity research. In order to get a thorough understanding of the ef-

fects of atmospheric aerosol on human health and environment, a panoramic study should be carried out since a single aerosol particle may carry multiple substances at the same time.

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REFERENCES

ANDERSEN A.A., (1958) — *New sampler for the collection, size and enumeration of viable airborne particles.* J. Bact., **76**:478-482.

BOVALLIUS A. ET AL., (1978) — *Three-year investigation of the natural airborne bacterial flora at four localities in Sweden.* Appl Environ. Microbiol., **35**:847-852.

BUCHANAN R.E., GIBBONS N.E., (1974) — *Bergey's manual of determinative bacteriology.* The 8 edition. The Williams & Wilkins.

CHE FENGXIANG ET AL., (1990) — *Ecological distribution of atmospheric bacteria over Beijing and Tianjin Area.* China Environmental Sciences, **10**:192-195.

HOLT J.G. ET AL., (1984, 1986) — *Bergey's manual of systematic bacteriology.* The 9 edition, Volume 1 (1984), Volume 2 (1986), The Williams & Wilkins.

LEE R.K. ET AL., (1973) — *Relationship between viable bacteria and air pollution in an urban atmosphere.* Am. Ind. Hyg. Assoc. J., **34**:164-170.

IMSHENETSKY A.A. ET AL., (1978) — *Upper Boundary of the biosphere.* Appl. Environ. Microbiol., **35**: 1-5.

JONES B.L. ET AL., (1983) — *Natural atmospheric microbial conditions in a typical suburban area.* Appl. Environ. Microbiol., **45**:919-934.

HU QINGXUAN ET AL., (1990) — *Size distribution of airborne microorganism particle.* China Environmental Science, **10**:99-102.

MANCINELLI R.L. ET AL., (1978) — *Airborne bacteria in an urban environment.* Appl. Environ. Microbiol., **35**:1095-1101.

THE GROUP OF CLASSIFICATION OF BACTERIA, INSTITUTE OF MICROBIOLOGY, ACADEMY OF SCIENCES OF CHINA, (1978) — *Methods for identification of general bacteria.* Scientific Press, Beijing.

TONG YONGYI ET AL., (1990) — *The effect of nitrogen dioxide on the survival of the aerosols of* Serratia marcescens *and* Bacillus subtilis *spore.* China Environmental Science, **10**:30-34.

WRIGHT J.J. ET AL., (1969) — *Viable microorganisms in an urban atmosphere.* J. Air. Pollut. Control. Assoc., **19**:337-341.

XI SUMING ET AL., (1988) — *The constitution of atmospheric bacteria. I. Species of atmospheric bacteria over Beijing and Tianjin area.* Acta Environ. Sci., **8**:39-47.

Photochemistry and Photobiology, 1997, 65(1): 103–106

EXHIBIT 6

Research Note

Solar Radiation Is Shown to Select for Pigmented Bacteria in the Ambient Outdoor Atmosphere

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ABSTRACT

The percentage of pigmented to total bacteria in the outdoor atmospheric population was studied in the field and in controlled laboratory experiments to evaluate the effects of solar radiation (SR) on bacterial survival. The field experiments showed that the percentage of pigmented bacteria positively correlated with SR activity during clear summer days. The percentage was lowest during darkness before dawn and around midnight (*ca* 33%) and as the SR increased during the day, gradually increased to a maximum of *ca* 50–60% at noontime to early afternoon and decreased thereafter. In the laboratory the ambient outdoor atmospheric bacteria impacted on culture plates were exposed to simulated SR and a germicidal light. With increased exposure, more nonpigmented bacteria were killed and the percentage of pigmented bacteria gradually increased. These observations suggest an inverse relationship between the atmospheric bacterial survival and the percentage of pigmented bacteria contained therein, thus supporting the notion that pigmentation might provide protection for outdoor atmospheric bacteria from sunlight damage. As a consequence, viable pigmented bacteria (and other UV-resistant forms) in the atmosphere could be enriched under areas of stratospheric ozone depletion.

INTRODUCTION

Pigmentation (*i.e.* pigmentation is colonial color other than white or clear) or some outdoor atmospheric bacteria has been thought to protect them from damaging solar radiation (SR)† (1,2). Imshenetsky *et al.* reported that five of the six strains of the microorganisms collected from the mesosphere (48–77 km above ground level) where intense damaging SR would be expected were pigmented. Pigmented bacteria are also abundant in the atmospheric boundary layer (ABL) where Tong *et al.* (2) found that approximately 65% of them were pigmented, including many in the predominant genera *Staphylococcus, Listeria* and *Micrococcus.* Although no detailed analysis has been made to analyze the chemical composition of these pigments, their yellow and orange colors resemble carotenoids (2). Carotenoids were found to protect microorganisms from near UV and visible light damage by quenching triplet-state photosensitizers and reactive oxygen species (3–6).

In this paper, we describe the proportion of pigmented bacteria found in the outdoor atmosphere that are affected by SR exposure. In supportive laboratory experiments we used simulated SR and germicidal light to irradiate the outdoor atmospheric bacteria collected on cloudy summer days. We hypothesized that the cloudy day bacteria would be less damaged due to SR absorption by cloudy vapor and therefore better subjects to evaluate the potential selective effects of SR on different atmospheric bacterial populations. We hope this study will help to elucidate the relationship between SR and pigmentation of natural atmospheric bacterial populations and the potential disturbance effect of the UVB radiation increase due to stratospheric ozone depletion on the structure of atmospheric bacterial populations (7,8).

MATERIALS AND METHODS

Sampling atmospheric bacteria. To quantify the diurnal distribution of pigmented bacteria, we collected ambient outdoor atmospheric bacteria on a rooftop (approximately 5 m above ground level) location 3 miles south of Corvallis, OR. The location had a large grass field to the southwest and a deciduous (*i.e.* oak, maple and ash) and evergreen (*i.e.* Douglas fir) mixed forest nearby in all other directions. Sample collections were made with slit samplers (S-T-A Biological Air Sampler, New Brunswick Scientific Co., Edison, NJ) arranged in a line perpendicular to the prevailing wind. Airborne bacterial particles were drawn through the slit of the sampler by a vacuum and impacted onto a rotating Luria–Bertani (LB) agar plate sitting on the sampler's turntable. Samplers were run concurrently at 47.5 L/min for 20 min. Samples for the temporal experiments to determine the pigmented bacteria in the atmosphere were collected on four clear summer days (3, 4, 5 and 21 August 1995) every 2 h from 0500 to 0900 h and from 1500 to 2300 h, and hourly from 0900 to 1500 h.

For simulated SR exposure experiments, we used bacterial samples collected on the rooftop location at noontime on a cloudy sum-

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†*Abbreviations:* ABL, atmospheric boundary layer; CFU, colony-forming units; LB, Luria–Bertani; %S, percent survival; SR, solar radiation; SSR, simulated solar radiation.

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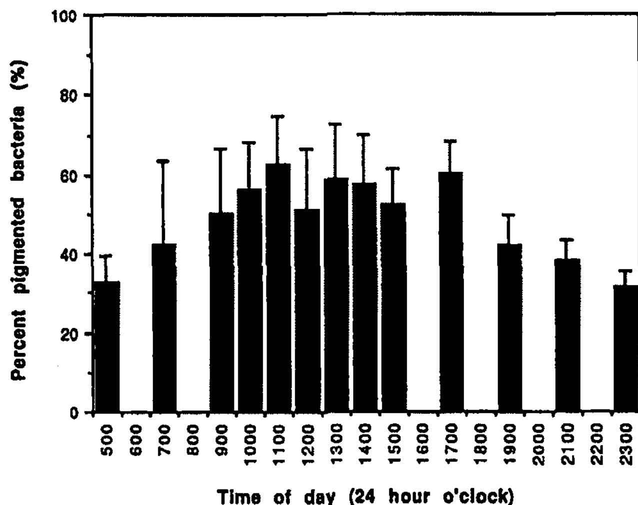

Figure 1. Mean diurnal change in the percentage of pigmented bacteria in the atmospheric bacterial population during 4 clear summer days in 1995 at Corvallis, OR. Two plates were used at each sampling time of a day.

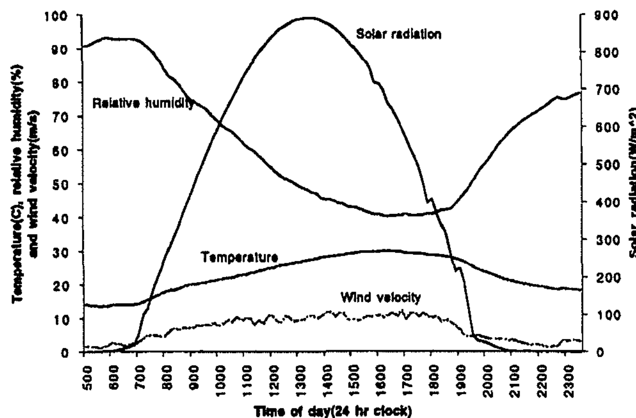

Figure 2. Mean weather conditions for 4 clear summer sampling days in 1995 at Corvallis, OR.

mer day, September 1995, from 1030 to 1530 h. For germicidal light exposure experiments, samples were taken from 1700 to 1900 h on a number of cloudy days in August and September of 1995.

To minimize any unwanted light effects, the slit sampler bonnets were covered with aluminum foil and duct tape. Also to prevent photoreactivation and other sunlight effects on the bacteria after collection, we removed the petri dishes from the samplers in a dark chamber and placed them in dark boxes. Before loading the petri dishes into the samplers, the slit barrel, bonnet and turntable of samplers were sterilized with 70% ethanol.

The agar (Difco Laboratories, Detroit, MI) amended with cycloheximide (200 μg/mL; Sigma Chemical Co., St. Louis, MO) was used as the culture media. The samples were incubated in the dark for 7 days at 25°C after which the total and pigmented bacteria were counted using a colony counter (Spiral System Inc., Cincinnati, OH). Pigmented bacteria were those considered yellow, orange and red.

Environmental conditions of temperature, relative humidity, wind direction, wind velocity and solar irradiance (400–1100 nm) were recorded using meteorological detectors attached to a 3 m tower (Campbell Scientific Inc., Logan, UT) located beside the sampling station. The meteorological instrumentation has been described previously (9).

Exposure of the bacteria to simulated solar radiation (SSR) and germicidal light. Two sources of radiation were used to expose the collected natural populations of atmospheric bacteria: (1) a solar simulator (model 8160, Oriel Corporation, Stratford, CT) and (2) a 25 W germicidal lamp (model G25T8, 18 inch long, GE Lighting Co., Cleveland, OH).

A 300 W xenon lamp solar simulator (model 8160, Oriel Corporation) with its airmass zero filter (termed AM0) was used to simulate the SR above the atmosphere, which was thought to represent the worst stratospheric ozone depletion situation. A recently factory calibrated radiometer (model IL440, International Light Co., Newburyport, MA) was used to measure the solar irradiance on the exposure area. Ultraviolet (250–400 nm) and total irradiance (250–1100 nm) on the exposure area were 53 and 1187 W/m^2, respectively. Because the output area of the solar simulator was smaller than the petri dish, only the colonies in the exposed region of the petri dish were counted. Exposure times for the collected populations of atmospheric bacteria were 0, 5, 15, 35, 75 and 120 min. Although the laboratory air was filtered throughout the experiments, we still placed a room-air fallout control petri dish beside the exposed plate during the irradiance exposure in case there was any abnormal contamination. A preliminary study showed that agar temperature increased to 42–43°C during SSR exposure. To compensate for the temperature effect on airborne bacterial survival, we placed the cloudy day collected outdoor atmospheric bacteria on the petri dish in a water bath set at 42–43°C and worked out a temperature-specific survival rate for each exposure time (data were not shown here).

The germicidal lamp was fixed on the inside top of a box with a blackened interior. The petri dishes with the collected atmospheric bacteria were positioned 18 cm from the light where the light intensity variation over the plate was <6%. The plates were exposed for 1 min. Because the exposure was carried out in an air-filtered clean laboratory, and exposure time was short (1 min), room-air fallout controls were thought to be unnecessary in this experiment. After irradiation exposure, the petri dishes were placed in a dark incubator for 7 days at 25°C.

To avoid photoreactivation effects due to stray light during manipulations, a 15 W tungsten light covered by a straw-colored filter (P39380, Edmund Scientific Co., Barrington, NJ) was used to illuminate the laboratory. This filter removes the photoreactivating light (<500 nm) and is a safe laboratory illuminating light source (10).

Analysis. The percentage of pigmented bacteria was the quotient of the pigmented bacterial colony-forming units (CFU), and total bacterial CFU on the same petri dish. For SSR and germicidal light exposure experiments, the percentages at different exposure times were normalized to the zero exposure time control.

Percent survival (%S) was calculated as the ratio of the CFU at different exposure time and the CFU at zero exposure time (control). We did not account for the room-air fallout control during SSR exposure because it was inconsequential (0–6 CFU, depending on the exposure time; if sunlight effect on the control plate was considered, it was even smaller) compared with the exposed bacteria collected on each plate (100–500 CFU). If the background bacterial CFU at an exposure time was unusually high (*i.e.* >10), the data were not used in the analyses. Further, to compensate for the temperature effect (42–43°C) during SSR exposure, we divided the %S by the temperature-specific survival (%) at that exposure time. Statistical test was done using SAS software (version 6.04).

RESULTS

Diurnal change of pigmented bacteria

The average percentages of pigmented bacteria in the outdoor atmospheric population from early morning (before sunrise) until midnight for 4 clear summer days are shown in Fig. 1. The data showed that the percentage was lowest at dawn and midnight (*e.g.* 33% and 32%, respectively). As the sun rose, the percentage gradually increased to a plateau of 50–64% from 0900 to 1700 h after which it gradually decreased to 32% at 2300 h. The average weather conditions during these periods are shown in Fig. 2. A Duncan statistical test showed that the pooled pigmented bacterial percentages during darkness (*e.g.* 0500, 0700, 2100 and 2300

Table 1. Effect of the SSR on the %S of the total and the pigmented bacteria from the outdoor atmosphere collected on a cloudy summer day in 1995 at Corvallis, OR

Exposure time (min)	Total irradiance (250–1100 nm, J/cm^2)	Total bacterial survival (n = 6) %	STDS	Percentage of pigmented bacteria (n = 5) Percentage of pigmented bacteria (%)	STDS
0	0.0	100.0	0.0	100.0	0.0
5	35.6	92.7	12.4	119.8	24.3
15	106.8	74.6	20.3	145.0	35.5
35	249.3	55.7	20.0	175.2	26.1
75	534.2	26.4	13.0	201.6	52.1
120	854.6	16.3	11.0	195.7	39.0

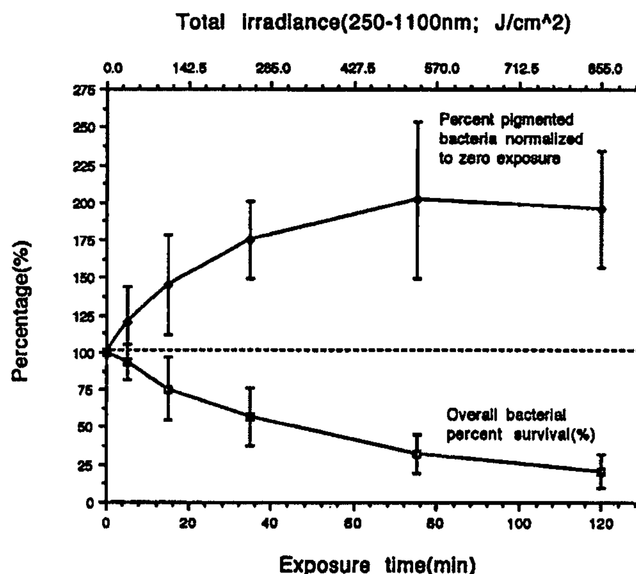

Figure 3. Effect of the SSR on the percent survival of the total and the pigmented bacteria from the outdoor atmosphere collected on a cloudy summer day in 1995 at Corvallis, OR. Data points are the means of six replications for the total bacterial survival and five for the percentage of the pigmented bacteria.

h) were significantly different ($P < 0.05$) from the percentages from 0900 to 1900 h when SR was relatively strong.

Lethal effect of the SSR and germicidal light on the pigmented bacteria

The SSR exposure of outdoor atmospheric bacteria collected on overcast summer days produced a decrease in survival of the overall bacterial population but an increase in the proportion of pigmented survivors (Table 1, Fig. 3). It appears there is an inverse relationship between overall bacterial survival and the percentage of pigmented bacteria due to SSR exposure.

Similarly, the exposure of the outdoor bacteria to the germicidal light for 1 min differentially killed *ca* 65.0 ± 3.5% (n = 6) of the bacteria with a resulting increase in the pigmented bacterial percentage to 169.8 ± 26.7% over the unexposed control (data not shown).

DISCUSSION

The percentage of pigmented bacteria in the outdoor atmospheric population appeared to correspond directly to the diurnal solar cycle (Figs. 1, 2). Although temperature, relative humidity and wind speed changed during the diurnal cycle (Fig. 2), it is theorized that SR is largely responsible for the population changes. To support this hypothesis, we exposed outdoor atmospheric bacteria collected on cloudy days to SSR and germicidal light. These experiments showed that there is a positive correlation between SSR exposure dosage and percent pigmented bacteria in the outdoor atmospheric populations (Table 1, Fig. 3). It is concluded that pigmented bacteria have greater resistance to both the sunlight and germicidal UV. The pigments of the outdoor atmospheric bacteria are primarily yellow, orange and sometimes red. Although we did not carry out analyses of these pigments, they appear to resemble carotenoids and function in a similar manner. Carotenoids were found to protect microorganisms from UV and visible light damage by quenching triplet-state photosensitizers and reactive oxygen species (3–6). We believe that the same pigments might be present in airborne bacteria and protect them from SR damage. Thus, an explanation of the observed diurnal cycle of the changing proportion of pigmented bacteria in the atmosphere could be due to the differential protection of yellow, orange and red pigments that are present in some atmospheric bacteria (*i.e.* more protected) but not in others (*i.e.* less protected).

Microorganisms exist in the atmospheric boundary layer to a height of at least 77 km (1,2,11). Although these microorganisms are mostly present as particles of single or clumped cells and/or "rafted" cells on debris (Lighthart and Paterno, unpublished electron micrographs) (12,13), compared to microorganisms in other environments, they are basically unprotected in the atmosphere. Therefore, they may be very easily damaged in the presence of SR. In response to pervasive and damaging SR, we assume that they have developed protective mechanisms. Except for pigmented bacteria, spore-forming bacteria are the only other prevalent atmospheric bacteria that are well known for their resistance to UV damage (2). We postulate that pigmentation and/or sporulation are important SR protection mechanisms for the outdoor atmospheric bacteria. These mechanisms could be developed either in their original well-exposed sunny habitats, such as on plant leaves or soil surfaces (14), or through recurrent airborne exposure that selects for enhanced resistance to sunlight damage.

If stratospheric ozone depletion progresses in the future, more solar UVB will penetrate the atmosphere to earth's surface (7,8). We speculate that pigmented (and perhaps spore-forming) bacteria will become more prevalent in the atmosphere below an ozone hole. This could also happen on other well-exposed, sunlit environments such as plant leaves where yellowish bacteria are already abundant (14,15). The consequences of a shift in the proportion of pigmented (and spore-forming) bacteria in these environments may be problematical and should be researched further.

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REFERENCES

1. Imshenetsky, A. A., S. V. Lysenko and G. A. Kazakov (1978) Upper boundary of the biosphere. *Appl. Environ. Microbiol.* **35**, 1–5.
2. Tong, Y., F. Che, X. Xu, M. Chen and B. Ye (1993) Population study of atmospheric bacteria at the Fengtai district of Beijing on two representative days. *Aerobiologia* **9**, 69–74.
3. Margalith, P. Z. (1992) *Pigment Microbiology*, pp. 32–76. Chapman & Hall, London.
4. Turveson, R. W., R. A. Larson and J. Kagan (1988) Role of cloned carotenoid gene expressed in *Escherichia coli* in protecting against inactivation by near UV light and specific phototoxic molecules. *J. Bacteriol.* **170**, 4675–4680.
5. Blanc, P. L., R. W. Turveson and M. L. Sargent (1976) Inactivation of carotenoids-producing and albino strains of *Neurospora crassa* by visible light, blacklight and ultraviolet radiation. *J. Bacteriol.* **125**, 616–625.
6. Gascon, J., A. Oubina, A. Perez-Lezaunand and J. Urmeneta (1995) Sensitivity of selected bacterial species to UV radiation. *Curr. Microbiol.* **30**, 177–182.
7. Frederick, J. E., H. E. Snell and E. K. Haywood (1989) Solar ultraviolet radiation at the earth's surface. *Photochem. Photobiol.* **50**, 443–450.
8. Kerr, J. B. and C. T. McElroy (1993) Evidence for large upward trends of ultraviolet-B radiation linked to ozone depletion. *Science* **262**, 1032–1034.
9. Lighthart, B. and B. T. Shaffer (1994) Bacterial flux from chaparral into the atmosphere in midsummer at a high desert location. *Atmos. Environ.* **28**, 1267–1274.
10. Harm, H. (1976) Repair of UV-irradiated biological systems: photoreactivation. In *Photochemistry and Photobiology of Nucleic Acids*, Vol. 2, *Biology* (Edited by S. Y. Wang), pp. 219–263. Academic Press, New York.
11. Fulton, J. D. (1966) Microorganisms of the upper atmosphere. 2. Relationship between altitude and micropopulation. *Appl. Environ. Microbiol.* **14**, 237–240.
12. Lighthart, B., B. T. Shaffer, B. Marthi and L. M. Ganio (1993) Artificial wind-gust liberation of microbial bioaerosols previously deposited on plants. *Aerobiologia* **9**, 181–186.
13. Lighthart, B. and B. T. Shaffer (1995) Viable bacterial aerosol particle size distributions in the midsummer atmosphere at an isolated location in the high desert chaparral. *Aerobiologia* **11**, 19–25.
14. Goodfellow, M., B. Austin and C. H. Dickinson (1976) Numerical taxonomy of some yellow pigmented bacteria isolated from plants. *J. Gen. Microbiol.* **97**, 219–233.
15. Dickinson, C. H. (1986) Adaptations of microorganisms to climatic conditions affecting aerial plant surfaces. In *Microbiology of the Phyllosphere* (Edited by N. J. Fokkema and J. van den Heuvel, Jr.), pp. 77–100. Cambridge University Press, Cambridge.

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EXHIBIT 7

Vol. 147, No. 3

Pigments of *Staphylococcus aureus*, a Series of Triterpenoid Carotenoids

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The pigments of *Staphylococcus aureus* were isolated and purified, and their chemical structures were determined. All of the 17 compounds identified were triterpenoid carotenoids possessing a C_{30} chain instead of the C_{40} carotenoid structure found in most other organisms. The main pigment, staphyloxanthin, was shown to be α-D-glucopyranosyl 1-*O*-(4,4′-diaponeurosporen-4-oate) 6-*O*-(12-methyltetradecanoate), in which glucose is esterified with both a triterpenoid carotenoid carboxylic acid and a C_{15} fatty acid. It is accompanied by isomers containing other hexoses and homologs containing C_{17} fatty acids. The carotenes 4,4′-diapophytoene, 4,4′-diapophytofluene, 4-4′-diapo-ζ-carotene, 4,4′-diapo-7,8,11,12-tetrahydrolycopene, and 4,4′-diaponeurosporene and the xanthophylls 4,4′-diaponeurosporenal, 4,4′-diaponeurosporenoic acid, and glucosyl diaponeurosporenoate were also identified, together with some of their isomers or breakdown products. The symmetrical 4,4′-diapo- structure was adopted for these triterpenoid carotenoids, but an alternative unsymmetrical 8′-apo- structure could not be excluded.

Rosenbach (26), in one of the first descriptions of pyogenic cocci, distinguished between the catalase-negative streptococci and the catalase-positive staphylococci and further subdivided the staphylococci into those which produced orange-yellow or "golden" colonies on appropriate culture media (*Staphylococcus aureus*) and those which produced white colonies (*Staphylococcus albus*). The division of the genus into species is now based on properties considered more reliable than pigment formation (4, 28), but the specific epithet *aureus* remains as a reminder of one of the most readily observed features of *S. aureus*, the characteristic color of its colonies. When freshly isolated from natural sources, most strains produce colonies which are orange in color (4); some, particularly those of bovine origin or those showing multiple resistance to antibiotics, may be yellow (45), whereas others, although being in other respects typical strains of *S. aureus*, produce no pigment ("white" strains).

There is general agreement that these orange and yellow pigments are carotenoids but, in spite of studies by a number of workers, there is considerable disagreement about their precise chemical structure. Chargaff (6) claimed, mainly on the basis of the electronic absorption spectra of extracted pigments, to have identified zeaxanthin (β,β-caroten-3,3′-diol) and zeaxanthin esters, and Allegra et al. (3) and Steuer (30) also considered the main pigment to be zeaxanthin. Other workers have reported the main components to be not zeaxanthin but other carotenoids, singly or in mixtures, such as δ-carotene (ε, ψ-carotene), rubixanthin (β,ψ-caroten-3-ol), and rubixanthin esters (10, 29, 31) or δ-carotene and sarcinaxanthin [2,2′-bis(4-hydroxy-3-methyl-2-butenyl) γ,γ-carotene; 9, 27]. In addition, various amounts of more reduced compounds such as phytoene (7,8,11,12,7′,8′,11′,12′-octahydro-ψ,ψ-carotene), ζ-carotene (7,8,7′,8′-tetrahydro-ψ,ψ-carotene), and phytofluenol-like compounds have also been reported (10) and are presumed to be biosynthetic precursors of the main pigments.

Marshall and Rodwell have already presented evidence (J. H. Marshall and E. S. Rodwell, 3rd Int. Symp. Carotenoids Abstr. Commun., p. 56-57, 1972) that the major pigment of orange-pigmented strains of *S. aureus* cannot be any of these compounds, but must be a previously undescribed structure, which was named staphyloxanthin. More recently, Taylor and Davies (R. F. Taylor and B. H. Davies, 4th Int. Symp. Carotenoids Abstr. Commun., p. 66-67, 1975) have reported briefly that the major xanthophyll of *S. aureus* (strain 209P) does not possess the common tetraterpenoid (C_{40}) carotenoid structure, but is a triterpenoid (C_{30}) carotenoid with the structure 4,4′-diaponeurosporen-4-oic acid (4,4′-diapo-7′,8′-dihydro-ψ,ψ-caroten-4-oic acid); there is also evidence that it may form esters and glycosides (7). This major xanthophyll is

accompanied by triterpenoid carotenes which, it has been suggested (8), may be intermediates of a pathway for triterpenoid carotenoid biosynthesis which is analogous to the Porter-Lincoln pathway for tetraterpenoid carotenoid biosynthesis (24, 25). "Bacterial phytoene," isolated by Suzue from a white mutant of *S. aureus* 209P, had already been identified as a C_{30} homolog of phytoene, and it was proposed that it is a precursor of C_{40} carotenoids in this organism, although the possibility that it is the precursor of other C_{30} carotenoids was not excluded (32, 33).

Triterpenoid carotenoids have been reported in certain other bacteria. Aasen et al. (1) obtained the triterpenoid carotenoid glycoside methyl 1-mannosyloxy-3,4-didehydro-1,2-dihydro-8′-apo-ψ-caroten-8′-oate from two organisms described as yellow halophilic cocci. Taylor and Davies (34, 35, 37) isolated and characterized a series of triterpenoid carotenes and xanthophylls from *Streptococcus faecium* UNH564P, a yellow-pigmented strain (recent work [40] argues in favor of classifying these pigmented streptococci as a separate species, *Streptococcus casseliflavus*). The main pigments produced by this organism in unaerated cultures were carotenes, whereas in aerated cultures it produced mainly the glucoside 4-D-glucopyranosyloxy-4,4′-diapo-7,8-dihydro-ψ,ψ-carotene (glucosyl-diaponeurosporenol; 38). *Halobacterium cutirubrum*, an organism which produces both C_{40} and C_{50} carotenoids, also produces a C_{30} phytoene (17).

We reexamined the nature of the pigments produced by *S. aureus*, and from the results reported here, we conclude that they are all triterpenoid carotenoids or derivatives of them, some possessing novel structures. In the accompanying paper (19), we present evidence for the pathway by which these pigments are produced biosynthetically. A preliminary report of this work has been given previously (J. H. Marshall and G. J. Wilmoth, 5th Int. Symp. Carotenoids Abstr. Commun., p. 36, 1978).

MATERIALS AND METHODS

Organisms and growth conditions. The organism used for most of this work was *S. aureus* S41, isolated originally in 1965 in Melbourne, Australia, and chosen initially because of its ability to produce strongly pigmented colonies. Its phage typing pattern is 52/52A/42E/83A/81/95.

A number of mutants with altered pigment patterns were derived from this strain and, in some cases, were more convenient sources of certain carotenoids than the wild-type strain; details of their isolation and properties are given in the accompanying paper (19). *S. aureus* strain FDA209P (NCTC 7447) and the neotype strain NCTC 8532 were obtained from the National Collection of Type Cultures, Colindale, England.

Organisms were normally maintained as freeze-dried cultures, except for certain mutants (19). Working stocks were maintained on nutrient agar or glycerol monoacetate agar at 4°C after overnight growth at 37°C. The use of glycerol monoacetate as a substrate supporting good pigment production was described by Willis and Turner (46). Glycerol monoacetate broth contained (per liter): tryptone (Oxoid Ltd., London, England), 10.0 g; yeast extract (Difco Laboratories, Detroit, Mich.), 2.5 g; glycerol monoacetate (Koch-Light), 6.0 ml; Tris (Sigma Chemical Co., St. Louis, Mo.), 12.0 g; nicotinic acid, 1.2 mg; thiamine hydrochloride, 0.4 mg; biotin, 0.002 mg; the final pH was adjusted to 7.0 to 7.2 before autoclaving at 121°C for 10 min. In some experiments, the Tris buffer in the medium was replaced by phosphate (Na_2HPO_4, 2.1 g; KH_2PO_4, 0.7 g). Solid medium was prepared by incorporating 1% agar in the medium. Cells for pigment studies were normally grown in glycerol monoacetate broth in wide-mouthed Erlenmeyer flasks, the medium occupying one-fifth of the flask volume. Flasks were inoculated from an 18-h broth culture (0.1 ml per 100 ml) and incubated on a rotary shaker (eccentric radius, 3.5 cm) at 160 rpm and 37°C for 24 to 40 h (conditions which ensure good aeration) or under similar conditions in an orbital incubator (Gallenkamp, model IH-400).

Dry weight determination. The dry weight of cells was determined by measurement of the absorbance of cell suspensions at 580 nm with a Spectronic 20 spectrophotometer (Bausch & Lomb, Inc., Rochester, N.Y.). The relation between dry weight and absorbance was linear up to an absorbance of 0.7; dry weight (milligrams per milliliter) = 0.27 × absorbance reading.

Chemicals. Analytical reagent grade chemicals were used where possible, failing which the purest commercially available grade was used. Light petroleum was Petroleumbenzin (b.p., 40 to 60°C; E. Merck, Darmstadt, W. Germany) unless otherwise specified. Most organic solvents were obtained from E. Merck or British Drug Houses (Poole, England) and used without further purification; where further purification was necessary, the methods described by Taylor and Davies (34) were used.

Extraction of carotenoids. Cells were harvested by centrifugation (5,000 × *g*, 10 min) and washed twice with water. The packed cells could be extracted immediately or stored at −20°C for up to 3 months without their carotenoid content being affected. They were suspended in methanol (40 ml per g [dry weight] of cells), heated in a water bath at 55°C for a few minutes while being stirred with a gentle stream of nitrogen, cooled, and centrifuged. The extraction was repeated if necessary until all pigment had been extracted. To avoid alteration of the carotenoids, manipulations were performed under nitrogen, exposure to bright light was avoided, and material was never stored in polar solvents. The combined methanol extracts were shaken with 1 volume of ethyl acetate and 3 volumes of 1.7 M aqueous NaCl, the ethyl acetate layer was removed, the aqueous layer if still colored was extracted with more ethyl acetate, and the combined ethyl acetate extracts were dried with anhydrous Na_2SO_4. The solvent was then removed in vacuo, the residue was dissolved in light petroleum-acetone (1:1,

vol/vol), and the solution was cooled to −20°C to precipitate phospholipids. For storage, it was preferable to replace this solvent mixture with light petroleum even though some of the polar carotenoids were not soluble in it.

Chromatographic methods. (i) Sephadex LH-20 columns. Sephadex LH-20 (Pharmacia, Uppsala, Sweden) was swollen before use by suspension in chloroform and then packed in a column (20 by 1 cm). Carotenoid extracts were transferred from light petroleum to chloroform solution. The chloroform solution was applied to the column, and the carotenoids were eluted with chloroform or, for more-polar components, a chloroform-methanol mixture.

(ii) Alumina columns. Alumina (Merck or Calbiochem; neutral, deactivated to Brockmann grade II) was suitable for separation of carotenes and low-polarity xanthophylls, but higher-polarity xanthophylls were difficult or impossible to elute. Some carotenoids, when left in contact with alumina, are degraded, and delays or interruption in the flow of solvent during separations should be avoided. Elution started with light petroleum, followed by light petroleum-acetone mixtures of increasing polarity, acetone, and in some cases acetone-methanol mixtures.

(iii) TLC. Thin-layer chromatography (TLC) was used as an analytical method and a preparative method. Plates were prepared by spreading silica gel (Kieselgel 60HR; Merck) on glass plates in 0.25-mm layers for analytical work or in 0.5-mm layers for preparative work and were activated at 110°C for 30 min. Silver nitrate-impregnated silica gel plates were prepared by incorporating 2.5% $AgNO_3$ and 5% concentrated NH_4OH in the suspending fluid for slurrying the silica gel (47). The following solvent systems were used: (i) light petroleum-acetone, 99:1 (vol/vol); (ii) light petroleum-acetone, 4:1 (vol/vol); (iii) light petroleum-acetone, 13:7 (vol/vol); (iv) benzene-methanol-acetic acid, 87:11:2 (vol/vol/vol). Spots or bands from thin-layer plates were recovered by scraping the appropriate portion of silica gel into a tube and eluting with light petroleum-acetone or acetone. Most carotenoids are readily detected as colored spots or bands; diapophytofluene, although colorless, fluoresces strongly in UV light. Colorless components were detected by exposure to iodine vapor, by spraying with sulfuric acid and heating, or by eluting and determining electronic absorption spectra.

(iv) GLC. Analyses by gas-liquid chromatography (GLC) were performed on a Perkin-Elmer model F-11 gas chromatograph (Perkin-Elmer, Beaconsfield, England). Glass columns (1.5 m by 4 mm) packed with 2% SE-52 on Gas-Chrom Q (80 to 100 mesh) were run isothermally at temperatures up to 330°C, using nitrogen as the carrier gas and a flame ionization detector. Many carotenoids are not stable under these conditions but can be converted into stable derivatives by hydrogenation over platinum oxide (36).

Spectroscopy. Electronic absorption spectra were determined with a Hitachi-Perkin-Elmer double-beam recording spectrophotometer (model 124; Perkin-Elmer, Norwalk, Conn.) calibrated against the 651.1-nm band of deuterium. Infrared spectra were determined in carbon tetrachloride solution with a Perkin-Elmer infrared recorder console (model 180); the "waxy" consistency of solid carotenoids made it difficult to compress them into a KBr pellet. Some initial determinations of mass spectra were made by G. P. Moss, Department of Chemistry, Queen Mary College, London, England; later determinations were made by S. Middleton, Department of Chemistry, Monash University, on a VG-Micromass 70/70F mass spectrometer, using a direct-probe insertion technique, a probe temperature of 200 to 220°C, and an ionizing potential of 70 eV.

Quantitative determination of carotenoids. Carotenoids were estimated quantitatively by measuring absorbance of solutions in light petroleum at appropriate wavelengths. Values for the specific extinction coefficients ($E^{1\%}_{1cm}$) of individual carotenoids were adapted from the molar extinction values of corresponding C_{40} carotenoids as described by Taylor and Davies (34). Values used for C_{30} carotenes are: 4,4′-diapophytoene (15-*cis*), 1,009 at 286 nm; 4,4′-diapophytofluene, 2,105 at 347 nm; 4,4′-diapo-7,8,11,12-tetrahydrolycopene, 3,367 at 395 nm; 4,4′-diapo-ζ-carotene, 3,415 at 400 nm; 4,4′-diaponeurosporene, 3,905 at 435 nm; 4,4′-diapolycopene, 4,450 at 466 nm. The $E^{1\%}_{1cm}$ value for 4,4′-diaponeurosporene was also used as a nominal value for its *cis* isomers and for the xanthophylls 4,4′-diaponeurosporenal (3,905 at 466 nm) and 4,4′-diaponeurosporenoic acid (3,905 at 455 nm). Corresponding values for the glycosides were: glucosyl-diaponeurosporenoate, 2,860 at 462 nm; staphyloxanthin, 1,920 at 462 nm.

Chemical characterization reactions. (i) Acetylation was used to determine the number and nature of hydroxyl functions (2). The carotenoid (50 to 500 μg) was dissolved in 1 ml of dry pyridine, 0.1 ml acetic anhydride was added, and the mixture was placed in the dark under nitrogen at room temperature for 12 to 24 h. Sodium chloride solution (5 ml, 1.7 M) was then added, the product was extracted into diethyl ether (two 5-ml volumes), and the combined extracts were washed with 1.7 M NaCl to remove pyridine; the solvent was then removed in vacuo. The acetylated product was dissolved in light petroleum and purified by TLC on silica gel with solvent iii. Primary, secondary, and tertiary alcohols are distinguished by their ease of acetylation (18); primary alcohols are fully acetylated within 10 to 30 min, secondary alcohols require 3 to 6 h for acetylation, and tertiary alcohols do not react. To determine whether tertiary hydroxyls were present, the fully acetylated product was further treated with dry pyridine and bis(trimethylsilyl)-acetamide (Pierce Chemical Co., Rockford, Ill.), and the product was recovered and purified as for acetyl derivatives. Tertiary hydroxyls are silylated under these conditions (13).

(ii). Sodium borohydride reduces aldehydes and ketones but not carboxylic acids or their esters (2, 18), but some carotenol esters may be saponified (12). An ethanolic solution of carotenoid was treated with a few crystals of $NaBH_4$ and placed in the dark at room temperature for 60 min; 1.7 M NaCl was then added, and the product was extracted into diethyl ether and purified.

(iii) Lithium aluminum hydride reduces aldehydes, ketones, and esters to hydroxyl derivatives (2). To the carotenoid dissolved in dry diethyl either, $LiAlH_4$ (1

to 2 mg) was added and allowed to react at room temperature for a few minutes. The reaction was terminated by the addition of a few milliliters of wet ether followed by 5 ml of 1.7 M NaCl, and the product in the ether phase was concentrated and purified. In some cases, difficulties were encountered in isolating products from strongly polar xanthophylls owing to their adsorption on the alumina precipitate.

(iv) Saponification with methanolic KOH is a standard procedure for preliminary purification of carotenoids in which it is assumed that not only glycerol esters but also any carotenol- or carotenoic acid-containing esters will be hydrolyzed to free acids. Taylor and Davies, however, have shown that 4% KOH at room temperature produces rapid transesterification of carotenoic esters or esterification of free carotenoic acids to the corresponding methyl esters (7; Taylor and Davies, 4th Int. Symp. Carotenoids Abstr. Commun.). More concentrated alkali (10% KOH) acting for 12 to 24 h is needed to ensure hydrolysis of esters (including methyl esters) to free acids. Esters can also be hydrolyzed to free acids with no possibility of methyl ester formation by treatment with 5% KOH in acetone at room temperature for 60 min; the alkaline acetone solution should be freshly prepared to keep to a minimum the formation of alkali-catalyzed polymerization products of acetone, which do not then interfere.

(v) Carotenoid isomerization was performed by the Zechmeister method, employing UV irradiation in the presence of iodine (34).

(vi) Partition coefficients between light petroleum and 95% methanol were determined by the method of Petracek and Zechmeister (21).

Identification of sugar moiety of carotenoid glycosides. The carotenoid glycoside (100 to 500 μg) was hydrolyzed in HCl-saturated chloroform as described by Taylor and Davies (35), and the water-soluble fraction was used for chromatographic and enzymatic analyses for carbohydrate. Chromatographic analysis was by TLC on Kieselguhr G plates buffered with phosphate at pH 5 and eluted with butan-1-ol–acetone–0.05 M phosphate (pH 5; 4:5:1, vol/vol/vol) (41). Sugars were detected by spraying the plates with $AgNO_3$ solution followed by NaOH solution and heating (16). Enzymatic analyses for glucose employed Glucostat reagent (glucose oxidase-peroxidase reagent; Worthington Diagnostics, Freehold, N.J.).

Identification and estimation of fatty acids. Material containing fatty acids was treated with methanol-boron trifluoride; the resulting fatty acid methyl esters were extracted into diethyl ether and analyzed by GLC in the system described previously, using a column temperature of 175°C. Quantitative estimation was by comparison of peak areas with those of known standards. Reference compounds included the saturated *n* acids C_{13}, C_{14}, C_{15}, C_{16}, C_{17}, and C_{18} as well as the branched-chain anteiso- acids C_{15} and C_{17} prepared from *S. aureus* lipids.

Glycerol determination. Glycerol was measured enzymatically by using glycerol kinase and NAD-dependent glycerol 3-phosphate dehydrogenase (Boehringer Mannheim).

Radioactivity measurements. Activity of ^{14}C-containing material was measured with a liquid scintillation spectrometer (model 2002; Packard Instrument Co., Rockville, Md.). The scintillation fluid was prepared by dissolving 5 g of 2,5-diphenyloxazole [PPO] and 0.1 g of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)] benzene in 1 liter of toluene and adding 500 ml of Triton X-100. Carotenoid samples were bleached with benzoyl peroxide before being counted (42). [^{14}C]acetic anhydride was obtained from the Radiochemical Centre, Amersham, England.

RESULTS

Extraction and purification methods. Brief treatment of wet-packed cells with warm methanol (43) proved the most satisfactory of the methods tried for extracting pigments from *S. aureus*. Such extracts usually contain, in addition to carotenoids, other polyisoprene compounds such as squalene, menaquinones, and bactoprenol (undecaprenol and homologs) as well as phospholipids and glycolipids. It is customary to remove saponifiable lipids either by incorporating alkali in the extracting solvent or by a subsequent saponification step with methanolic KOH (18). With *S. aureus* extracts, saponification must be avoided; otherwise some xanthophylls, which are rapidly and irreversibly changed by dilute alkali, will be lost (Marshall and Rodwell, 3rd Int. Symp. Carotenoids Abstr. Commun.). Phospholipids can be removed by an alternative method involving precipitation by cold acetone (see above).

For purification and isolation of individual carotenoids, a combination of chromatographic methods was used. Chromatography on Sephadex LH-20 columns was the most useful system for initial fractionation of crude extracts; elution with chloroform followed by chloroform-methanol separated four main fractions and permitted complete recovery of all material from the column (Table 1). By suitable adjustment of the column size and flow rate of the solvent, fraction 3 could be resolved into two separate bands (diaponeurosporenoic acid and staphyloxanthin), but it was usually preferable to use other chromatographic systems to resolve each of fractions 1 to 3 into individual components. Individual carotenoids isolated from *S. aureus*, together with their spectral characteristics and order of elution from alumina (grade II) columns by light petroleum and light petroleum-acetone, are listed in Table 2. Alumina gave good resolution of fraction 1 but was less satisfactory for chromatography of more polar components, some of which became irreversibly bound to alumina and could not be eluted. There was also evidence of breakdown of some components, the extent being proportional to the time they were in contact with alumina.

TLC on silica gel with different solvent systems was capable of separating most of the carotenoids (Table 3), although it did not separate carotenes as well as did alumina columns. It was particularly useful when only limited amounts of material were available, for rapid qualitative analysis of crude extracts or fractions, and as a final purification step for some components. For example, up to 350 μg of staphyloxanthin could be separated as a narrow band without trailing on a 20-cm by 10-cm plate, 0.5 mm thick.

Carotenes. The hydrocarbon fraction contained, in addition to squalene (20 μg/g [dry weight]), several carotenes which appeared to correspond to those first isolated by Taylor and Davies (34) from *Streptococcus faecium* and later reported by them to be also present in *S. aureus* 209P (4th Int. Symp. Carotenoids Abstr. Commun.). The chromatographic properties, epiphasic behavior (partition ratio, 100:0), and absorption maxima are in good agreement with those reported here (Table 2). Upon hydrogenation and analysis by GLC (Table 4), the derivatives all ran with the same retention time as squalane, indicating that they were all C_{30} compounds; mass spectral determinations on the staphylococcal diapophytoene (*m/e*, 408, corresponding to a molecular formula of $C_{30}H_{48}$) and

TABLE 1. *Chromatography of carotenoids and related compounds of S. aureus on Sephadex LH-20*

Fraction	Eluted by:	Composition
1	Chloroform	Squalene Carotenes Menaquinone Carotenals
2	Chloroform	Bactoprenol Isostaphyloxanthin Hydroxy-400 compounds
3	Chloroform-methanol (99:1)[a]	Diaponeurosporenoic acid Staphyloxanthin
4	Chloroform-methanol (19:1)[b]	Glucosyl-diaponeurosporenoate

[a] Chloroform alone will slowly elute fraction 3.
[b] Increasing methanol concentration causes swelling of Sephadex, and the flow rate decreases considerably.

TABLE 2. *Chromatography on alumina and identity of carotenoids isolated from S. aureus*

Chromatography[a] Band	Chromatography[a] Solvent	Color	λ_{max} (nm) in light petroleum[b]					Identification	Carotenoid[c] content (μg/g [dry wt])
1	0	Colorless			—			Squalene	20
2	0	Colorless		275	286	298		15-*cis*-4,4′-Diapophytoene	40
3	0	Colorless (fluorescent)		330	347	366		4,4′-Diapophytofluene	3
4	0	Pale yellow		374	395	419		4,4′-Diapo-7,8,11,12-tetrahydrolycopene	2
5	0.5	Yellow		405	428	456		Neo-4,4′-diaponeurosporene C	1
6	0.5	Pale yellow		378	400	425		4,4′-Diapo-ζ-carotene	6
7	0.75	Yellow		407	430	459		Neo-4,4′-diaponeurosporene B	1
8	1	Yellow		412	435	465		4,4′-Diaponeurosporene (all-*trans*)	10
9	1	Red		440	466	498		4,4′-Diapolycopene	ND[d]
10	1.5	Colorless	243	248	260	269	325	Menaquinone	
11	3	Red	346	(441)	463	492		*cis*-4,4′-Diaponeurosporenal	ND
12	3	Red		(444)	466	496		4,4′-Diaponeurosporenal	1
13	3	Red		(454)	476	508		4,4′-Diapolycopenal	ND
14[e]	20	Pale yellow			—			Bactoprenol	
		Pale yellow		378	400	422	(445)	Hydroxy-400 compounds	10
15	30	Orange		—	460	(489)		Isostaphyloxanthin	10
16	40	Orange		—	462	(491)		Staphyloxanthin	360
NE[f]		Yellow		432	455	483		4,4′-Diaponeurosporenoic acid	38
NE		Yellow		(430)	453	481		*cis*-4,4′-Diaponeurosporenoic acid	2
NE		Orange		—	462	(491)		Glucosyl-4,4′-diaponeurosporenoate	10

[a] Chromatography on alumina (grade II) column. Bands eluted successively by light petroleum-acetone mixtures of increasing polarity; percent acetone indicated in solvent column. Relative positions of three noncarotenoid polyisoprenes also shown (bands 1, 10, and 14).
[b] Dashes indicate no peak; parentheses indicate point of inflection.
[c] Average figures for several experiments; cells grown with good aeration (see text) for 40 h at 37°C, yielding 2.5 to 3.0 mg (dry weight) of cells per ml of culture; total carotenoid content, ca. 500 μg/g (dry weight).
[d] ND, Not detected in the wild-type strain but found in some mutants (19).
[e] 4,4′-Diaponeurosporenol (λ_{max}, 412, 435, and 465) was never detected in *S. aureus*, but authentic material eluted in band 14.
[f] Not eluted even by polar solvents.

TABLE 3. *TLC of carotenoids, squalene, menaquinone, and bactoprenol from S. aureus*[a]

Compound	R_f value			
	i	ii	iii	iv
Squalene	0.87			
4,4'-Diapophytoene	0.80			
4,4'-Diapophytofluene	0.62			
4,4'-Diapo-7,8,11,12-tetrahydrolycopene	0.49			
Neo-4,4'-diaponeurosporene C	0.47	>0.85	>0.85	>0.90
4,4'-Diapo-ζ-carotene	0.47			
Neo-4,4'-diaponeurosporene B	0.45			
4,4'-Diaponeurosporene	0.39			
4,4'-Diapolycopene	0.36			
Menaquinone	0.25	0.71	0.81	0.82
cis-4,4'-Diaponeurosporenal	0.10	0.64	0.72	0.75
4,4'-Diaponeurosporenal	0.10	0.60	0.68	0.75
4,4'-Diapolycopenal	0.10	0.57	0.65	0.75
Bactoprenol		0.55	0.65	0.60
4,4'-Diaponeurosporenol		0.54	0.63	0.60
cis-4,4'-Diaponeurosporenoic acid		0.12	0.52	0.47
4,4'-Diaponeurosporenoic acid	0	0.12	0.49	0.45
Isostaphyloxanthin		0.07	0.43	0.40
Staphyloxanthin		0.05	0.38	0.36
Glucosyl-diaponeurosporenoate		0	0.05	0.25

[a] TLC on silica gel. For composition of solvents i through iv, see the text.

diaponeurosporene (*m/e*, 402, corresponding to a molecular formula of $C_{30}H_{42}$) provided conclusive evidence of their size. Under the growth conditions used, the total carotene content of the cells was about 60 μg/g (dry weight) (10 to 15% of the total carotenoids); however, some of the mutant strains (19) produced much higher proportions of carotenes and were a more convenient source for their isolation.

The main members of this carotene series are 4,4'-diapophytoene (4,4'-diapo-7,8,11,12,7',8',11',12'-octahydro-ψ,ψ-carotene), 4,4'-diapophytofluene (4,4'-diapo-7,8,11,12,7',8'-hexahydro-ψ,ψ-carotene), 4,4'-diapo-ζ-carotene (4,4'-diapo-7,8,7',8'-tetrahydro-ψ,ψ-carotene) 4,4'-diapo-7,8,11,12-tetrahydrolycopene (4,4'-diapo-7,8,11,12-tetrahydro-ψ,ψ-carotene), and 4,4'-diaponeurosporene (4,4'-diapo-7,8-dihydro-ψ,ψ-carotene), possessing systems of 3,5,7,7- and 9-conjugated double bonds, respectively (Fig. 1). Only one isomer of 4,4'-diapophytoene was isolated; its properties correspond to those of the 15-*cis* isomer (7), in contrast to the other members of the series, which were all-*trans* isomers. For 4,4'-diaponeurosporene, in addition to the *trans* isomer, which constituted 80 to 90% of the total, two *cis* isomers were also isolated, the neo B and neo C isomers. Each of the three isomers, when subjected to iodine-catalyzed photoisomerization, gave an equilibrium mixture of the

TABLE 4. *GLC of carotenoids of S. aureus*

Compound	Retention[a] time (min)	Retention time/retention time of squalane
Reference compound		
Squalene	1.50	1.43
Hydrogenation products of:		
Squalene (i.e., squalane)	1.05	1.00
Lycopene[b] (i.e., lycopersane)	5.00	4.76
β-Carotene	3.40	3.26
Compound from *S. aureus*		
4,4'-Diapophytoene	2.10	2.00
Hydrogenation products of:		
Squalene	1.05	1.00
4,4'-Diapophytoene	1.05	1.00
4,4'-Diapophytofluene	1.05	1.00
4,4'-Diapo-ζ-carotene	1.05	1.00
4,4'-Diaponeurosporene	1.05	1.00
4,4'-Diaponeurosporenol[c]	1.50	1.43
4,4'-Diaponeurosporenal	1.55	1.47
4,4'-Diaponeurosporenoic acid	1.55	1.47
4,4'-Diaponeurosporenoic acid methyl ester	1.70	1.61
Staphyloxanthin	No clear peaks	

[a] Column: 2 % SE-52 on Gas-Chrom Q run isothermally at 250°C.
[b] Prepared from tomatoes.
[c] Prepared by reduction of staphyloxanthin with $LiAlH_4$.

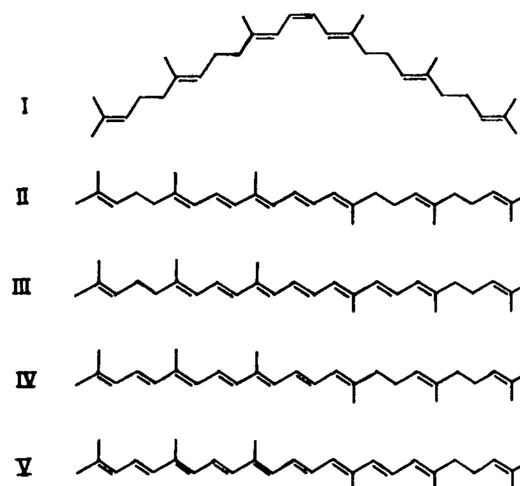

FIG. 1. *Triterpenoid carotenes of S. aureus. (I) 15-cis-4,4'-Diapophytoene; (II) 4,4'-diapophytofluene; (III) 4,4'-diapo-ζ-carotene; (IV) 4,4'-diapo-7,8,11,12-tetrahydrolycopene; (V) 4,4'-diaponeurosporene.*

trans (55%) and five *cis* isomers, neo A (2%), neo B (10%), neo C (30%), neo D (2%), and neo E (1%); the amounts found and positions of absorption maxima were in good agreement with reported values (34).

One novel carotene found in small quantities in certain mutants but not in the wild-type strain (21) was identified as 4,4′-diapolycopene (4,4′-diapo-ψ,ψ-carotene) on the basis of its absorption spectrum (λ_{max}, 440, 466, and 498 nm), which is identical to that reported for synthetic material prepared by dehydrogenation of squalene with *N*-bromosuccinimide (37). It has not previously been reported to occur naturally.

Carotenals. Carotenoid extracts from the wild-type organism contained a fraction present in small amounts (0.2% of the total) which could be separated as a red band (band 12; Table 2), but not in sufficient quantity for identification. Material sufficient for characterization was obtained from type IV mutants (21), for which it was the major carotenoid. Two isomers were found, separable by TLC with solvent iii, each having a partition ratio of 78:22 and being present in a ratio of approximately 4:1. The main all-*trans* isomer had λ_{max} values of (444), 466, and 496 nm in light petroleum, 467 nm in methanol, and 466 nm in acetone, whereas the values for the *cis* isomer in light petroleum were (441), 463, and 492 nm, with a prominent *cis* peak at 346 nm. Iodine-catalyzed photoisomerization of either gave an equilibrium mixture containing 67% *trans* isomer. The compound did not react with 4% methanolic KOH, could not be acetylated, and showed no acidic properties but was reduced by $NaBH_4$ or $LiAlH_4$ to a product with λ_{max} values (in light petroleum) at 413, 435, and 465 nm, which was identified as 4,4′-diaponeurosporen-4-ol. Hydrogenation gave a product, the retention time of which on GLC indicated that it had a C_{30} chain (Table 4). These properties are consistent with the identification of the compound as the aldehyde 4,4′-diaponeurosporen-4-al (4,4′-diapo-7′,8′-dihydro-ψ,ψ-caroten-4-al) described by Taylor and Davies (37) (Fig. 2, VI).

In extracts from type IV mutants, small amounts of a second aldehyde were present which ran as a faint purple band (band 13) closely following band 12 on alumina columns and was separable by repeated chromatography. Its properties were very similar to those of diaponeurosporenal but with λ_{max} values of (454) 476 and 508 nm, a partition ratio of 80:20, and a $NaBH_4$ reduction product having λ_{max} values of (444), 465, and 496 nm. Its properties suggest that it contains 11 conjugated double bonds, and it would appear to correspond to the compound identified by Taylor and Davies (37) as 4,4′-diapolycopen-4-al (4,4′-diapo-ψ,ψ-caroten-4-al).

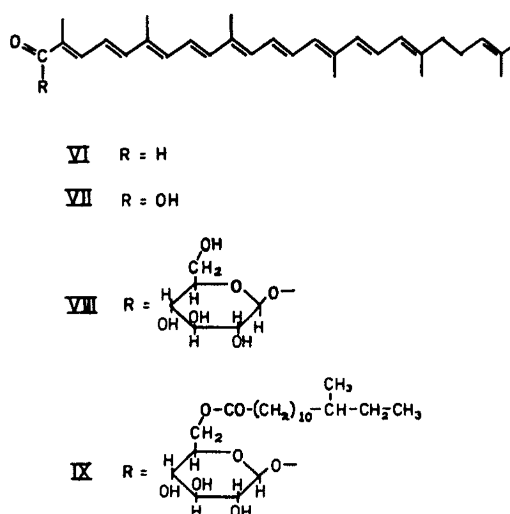

FIG. 2. *Triterpenoid xanthophylls of S. aureus. (VI) 4,4′-Diaponeurosporen-4-al; (VII) 4,4′-diaponeurosporen-4-oic acid; (VIII) glucosyl-diaponeurosporenoate; (IX) staphyloxanthin (α-D-glucopyranosyl 1-O-(4,4′-diaponeurosporen-4-oate) 6-O-(12-methyltetradecanoate).*

4,4′-Diaponeurosporen-4-oic acid. 4,4′-Diaponeurosporen-4-oic acid was obtained from crude extracts by preliminary fractionation on Sephadex LH-20 followed by TLC of fraction 3 with solvent iii; alumina columns could not be used, since the compound could not be eluted even by polar solvents. Some of its properties are listed in Table 5. The variation in the partition ratio with pH and its reduction to diaponeurosporenol by $LiAlH_4$ but not by $NaBH_4$ indicated that it was the carboxylic acid 4,4′-diaponeurosporen-4-oic acid (4,4′-diapo-7′,8′-dihydro-ψ,ψ-caroten-4-oic acid), which Taylor and Davies have reported to be the major xanthophyll of *S. aureus* 209P (Taylor and Davies, 4th, Int. Symp. Carotenoids Abstr. Commun.) (Fig. 2, VII). The behavior with methanolic KOH was unusual; 4% KOH at 20°C caused no detectable change over short periods (20 min), but longer exposure led to slow esterification (50% in 12 h); 10% KOH, however, favored hydrolysis, the ester being 95% hydrolyzed within 24 h. Esterification to the same product was also effected by methanol-BF_3. Acetylation with acetic anhydride-pyridine was slow, 40% of the acid being unchanged after 24 h, and a single product was formed which had the properties of a mixed anhydride; silylation also yielded a single product. The retention time of the hydrogenation product (Table 4), which, as with other xanthophylls, consisted of a mixture of hydrogenated xanthophyll and the corresponding hy-

TABLE 5. *Comparison of some properties of staphyloxanthin and 4,4'-diaponeurosporen-4-oic acid*

Compound	λ_{max}[a]			Partition ratio (light petroleum/95% methanol)	Reaction product
4,4'-Diaponeurosporenoic acid	432	455	483	10:90[b]	
	(422)	448	475[c]		
	(430)	455	(483)[d]		
Reaction with:					
4% Methanolic KOH	432	455	485	85:15	Methyl diaponeurosporenoate
10% Methanolic KOH	432	455	483	10:90[b]	No reaction
$NaBH_4$	432	455	483	10:90[b]	No reaction
$LiAlH_4$	413	435	465	50:50	Diaponeurosporenol
Acetylating agent		460		55:45	Acetyl anhydride
Silylating agent		460		55:45	Silyl derivative
cis Isomer	(430)	453	481	10:90[b]	
Staphyloxanthin		462	(491)	18:82	
		460	[c]		
		460	[d]		
Reaction with:					
4% Methanolic KOH	432	455	485	85:15	Methyl diaponeurosporenoate
10% Methanolic KOH	432	455	483	10:90[b]	Diaponeurosporenoic acid
4% KOH in acetone	432	455	483	10:90[b]	Diaponeurosporenoic acid
$NaBH_4$	432	455	483	10:90[b]	Diaponeurosporenoic acid
$LiAlH_4$	413	435	465	50:50	Diaponeurosporenol
Acetylating agent	(440)	463	492	55:45	Triacetate (see text)
Silylation of fully acetylated product	(440)	463	492	55:45	No silylation
cis Isomer	350	460		20:80	

[a] Spectra measured in light petroleum except where otherwise indicated. Parentheses indicate point of inflection.
[b] Partition ratio increased in dilute acid to 45:55 and decreased in dilute alkali to 0:100.
[c] Measured in methanol.
[d] Measured in acetone.

drogenated hydrocarbon, indicated a C_{30} chain.

The infrared spectrum showed a prominent carbonyl peak at 1,715 cm^{-1}, which disappeared after $LiAlH_4$ reduction, and a broad peak at 3,350 cm^{-1}, corresponding to a carboxylic hydroxyl which became more intense, moved to 3,400 cm^{-1} after $LiAlH_4$ reduction, and was not present in the methyl ester. Mass spectral data for the acid and its methyl ester were in close agreement with those reported by Davies (7), indicating a molecular weight for the acid of 432 and a molecular formula of $C_{30}H_{40}O_2$. These analytical data confirm the identification of the compound as 4,4'-diaponeurosporen-4-oic acid.

Staphyloxanthin. Under the conditions of growth used in this work, 70 to 80% of the total carotenoid produced by *S. aureus* S41 was staphyloxanthin. A comparison of some properties of staphyloxanthin and diaponeurosporenoic acid is made in Table 5. Their spectra differed considerably, the acid showing a three-peak spectrum, whereas staphyloxanthin showed a single broad peak at 460 nm in methanol or acetone and a broad peak at 462, with an inflection at 491 in light petroleum (Fig. 3). Treatment of staphyloxanthin with dilute alkali caused its spectrum to change rapidly to a three-peak spectrum, and the change could not be reversed by acidification; when 4% methanolic KOH was used, transesterification occurred, and the product was diaponeurosporenoic acid methyl ester; when 10% methanolic KOH, 4% KOH in acetone,

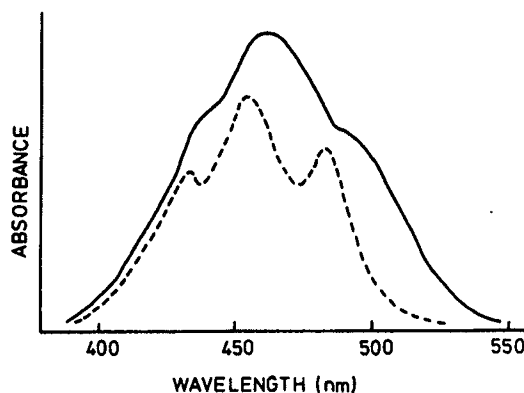

FIG. 3. *Electronic absorption spectra of xanthophylls. (——) Staphyloxanthin; (---) 4,4'-diaponeurosporenoic acid. Solvent, Light petroleum.*

or $NaBH_4$ was used, the product was the free acid.

Acetylation of staphyloxanthin with acetic anhydride-pyridine produced three separate products, the initial product reaching its maximum level within 40 to 50 min and then decreasing, the second reaching a maximum after about 120 min, and the third being the sole product after about 6 h. That each of these steps was sequential could be shown by isolating the first acetyl derivative by TLC (solvent iii; R_f, 0.51), subjecting it to further acetylation to produce the second derivative, isolating it (R_f, 0.62), and again acetylating to produce the fully acetylated product (R_f, 0.72). The presence of three acetylatable hydroxyl groups was confirmed by a quantitative measurement of the incorporation of ^{14}C into the products after acetylation of staphyloxanthin with [1-^{14}C]acetic anhydride, when the ^{14}C content of the three products corresponded to mono-, di-, and triacetyl derivatives, respectively (Table 6).

Earlier attempts to determine the structure of staphyloxanthin suggested that it was an ester of diaponeurosporenoic acid containing carbohydrate, fatty acid, and glycerol (Marshall and Wilmoth, 5th Int. Symp. Carotenoids Abstr. Commun.). Further work showed that these samples of staphyloxanthin were contaminated with glycolipid, which could not be separated by repeated TLC on silica gel with solvent iii or by TLC of acetylated derivatives in the same system. The presence of the glycolipid was shown by the blue color obtained after spraying with diphenylamine (16). Separation was achieved by TLC on silver nitrate-impregnated silica gel with solvent iii, in which system the R_f of staphyloxanthin was appreciably less than that of the glycolipid. Purified in this way it still contained diaponeurosporenoic acid, carbohydrate, and fatty acid, but no glycerol.

TABLE 6. *Determination of free hydroxyl groups with [1-^{14}C]acetic anhydride*

Carotenoid	Acetylated product[a] (cpm)	CH_3CO content[b] (nmol)	Carotenoid (nmol)	Acetyl/carotenoid ratio
Diaponeurosporenol	12,700	177	170	1.04
Staphyloxanthin				
First acetyl derivative	7,600	106	96	1.1
Second acetyl derivative	8,600	120	57	2.1
Third acetyl derivative	11,200	155	48	3.2
Isostaphyloxanthin	5,300	74	22	3.35
Glucosyl-diaponeurosporenoate	9,200	129	30	4.3

[a] Carotenoids were acetylated with [1-^{14}C]acetic anhydride-pyridine; values are for fully acetylated products, except for the first and second acetyl derivatives of staphyloxanthin.

[b] Specific activity of [1-^{14}C]acetic anhydride, 20.6 μCi/mmol.

The carbohydrate moiety was obtained in the water-soluble fraction after acid hydrolysis, and the main component had an R_f of 0.36 in the TLC system of Waldi (41), corresponding to glucose; about 5% each of two other carbohydrates was present with R_f values of 0.44 and 0.25, corresponding to mannose and galactose. The presence of glucose as the main component was confirmed by enzymatic assay with the Glucostat reagent.

Identification and measurement of fatty acids were carried out by GLC of their methyl esters after hydrolysis of the carotenoid with methanolic KOH. The main component was identified as the C_{15} anteiso- acid, 12-methyl-tetradecanoic acid; some of its C_{17} homolog 14-methyl-hexadecanoic acid was also present. Quantitative determinations of carotenoid, glucose, and fatty acid in staphyloxanthin yielded a ratio near 1:1:1, although the figure for glucose was always slightly less than one.

The infrared spectrum differs from that of methyl diaponeurosporenoate, mainly in showing, in addition to the carbonyl peak at 1,715 cm^{-1}, a second strong carbonyl band at 1,740 cm^{-1}, attributable to the fatty acid carboxyl, and a broad peak at 3,400 cm^{-1}, attributable to the glucose hydroxyls.

Mass spectra determinations on staphyloxanthin gave a pattern very similar to that of diaponeurosporenoic acid but with some additional peaks. The largest fragment detected was at *m/e* 432, corresponding to diaponeurosporenoic acid, with other peaks at *m/e* 415 (M-17) and 387 (M-45), indicating loss of —OH and —COOH. A prominent peak at *m/e* 242 accompanied by peaks at *m/e* 225 (M-17) and 197 (M-45) corresponded to a C_{15} monocarboxylic acid, whereas peaks at *m/e* 185 (M-57) and 57 were consistent with methylation at the third carbon,

giving the fragment $CH_3—CH_2—CH(CH_3)—$ (5). A small peak at *m/e* 269 indicated the presence of the homologous C_{17} fatty acid. Triacetylstaphyloxanthin gave a parent ion at *m/e* 944, corresponding to a molecular formula of $C_{57}H_{84}O_{11}$ (Fig. 4). Fragments at *m/e* 513 and 432 corresponded to splitting of diaponeurosporenoic acid from the rest of the molecule, whereas *m/e* 288 was the triacetylhexose moiety (M-432-225) and showed a typical fragmentation pattern of triacetylhexose (23), giving successive loss of acetyl groups producing peaks at *m/e* 229, 169, and 109; a small peak at *m/e* 703 corresponded to loss of fatty acid (M-242). A small peak was also de-

tected at m/e 972, attributable to the homolog containing a C_{17} fatty acid.

Staphyloxanthin thus appeared to be a derivative of diaponeurosporenoic acid linked by an ester linkage to glucose, which was also esterified with a C_{15} fatty acid. G. P. Moss (Queen Mary College, London, England) very kindly examined a sample of this material by mass and nuclear magnetic resonance spectrometry. He suggested that the carotenoid chain may have the unsymmetrical 8′-apo- structure, rather than the 4,4′-diapo- structure, and proposes for staphyloxanthin the structure α-D-glucopyranosyl 1-*O*-(8′-apo-ψ-carotenoate) 6-*O*-(12-methyl-tetradecanoate) (20), although we prefer the symmetrical 4,4′-diaponeurosporenoate for the carotenoid moiety (see Discussion). This structure is shown in Fig. 2 (IX). The relationship between the two alternative structures for the C_{30} chain, the 4,4′-diapo- and the 8′-apo-, and the C_{40} carotenoid chain are shown in Fig. 5. The analytical results indicate that staphyloxanthin is accompanied by small amounts of two isomeric forms in which glucose is replaced by mannose or galactose and by homologous forms containing the C_{17} fatty acid in place of the C_{15} fatty acid.

Isostaphyloxanthin. Material which ran just ahead of staphyloxanthin on Sephadex LH-

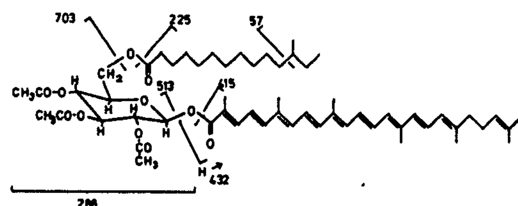

FIG. 4. *Mass spectrometry of triacetyl staphyloxanthin; derivation of main fragments.*

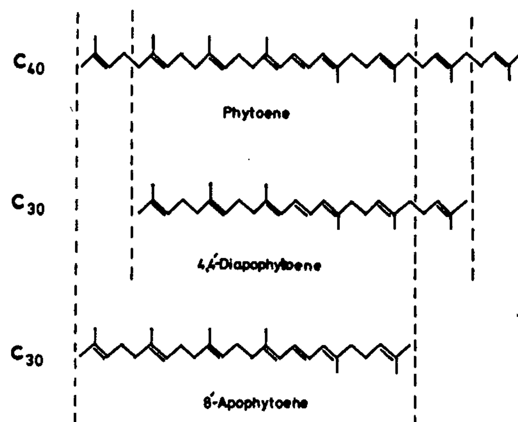

FIG. 5. *Relationship between alternative structures for "C_{30} phytoene" and phytoene.*

20 or alumina could be separated as a distinct fraction by TLC (solvent iii). When subjected to the tests listed in Table 5, it behaved like staphyloxanthin and differed from it only in having a slightly higher partition ratio (23:77) and λ_{max} values of 461 and (489) nm, the 489-nm inflection being slightly less prominent. It is not a *cis* isomer and, when subjected to iodine-catalyzed photoisomerization, showed hypochromic and hypsochromic changes in its spectrum to a λ_{max} of 459 nm and a *cis* peak at 357 nm. Stepwise acetylation and acetylation with [1-^{14}C]acetic anhydride (Table 6) showed the presence of three free hydroxyl groups. By the same methods as were used for staphyloxanthin, it was shown to contain diaponeurosporenoic acid, glucose, and a C_{15} fatty acid, although there was not sufficient material for a quantitative enzymatic assay for glucose. Its properties suggest that it is an isomer of staphyloxanthin which may differ only in the position of the ester linkages between glucose and the C_{15} and C_{30} chains.

Glucosyl-diaponeurosporenoate. After the structure of staphyloxanthin became clear, it seemed likely that its biosynthesis would proceed via an intermediate involving two of its three constituents. Such a compound was detected in the most polar fraction from the Sephadex LH-20 column (Table 1, fraction 4). This fraction also contained glycolipid and phospholipid, and purification required several separations by TLC on silica gel (solvent iv) followed by TLC of acetylated material on silver nitrate-impregnated silica gel (solvent iii). Hydrolysis of the pure material yielded diaponeurosporenoic acid and glucose (as shown by TLC and enzymatically). By the chemical tests used to characterize staphyloxanthin (Table 5), it was indistinguishable from it. Its spectrum was also indistinguishable from staphyloxanthin [λ_{max}, 462 and (491) nm], but it was more hypophasic (partition ratio, 2:98), and its more polar nature was apparent on chromatography. A total of five acetyl derivatives were detected during acetylation; determination of free hydroxyl groups by exhaustive acetylation with [1-^{14}C]acetic anhydride showed that there were four (Table 6). Only small amounts of material were available for analysis, which limited the accuracy of determination of the carotenoid/glucose ratio; the value obtained was 1:0.6. Its properties are consistent with it being a glucosyl ester of diaponeurosporenoic acid.

"Hydroxy-400" compounds. Trace amounts of a group of xanthophylls were isolated which all gave spectra similar to that of diapo-ζ-carotene but with an additional shoulder at 445 nm [λ_{max}, 378, 400, 425, and (445) nm]. More of this material was obtained from certain mu-

tant strains (19), which permitted limited investigation of this group. TLC (solvent iii) separated bands at R_f 0.50 and 0.55, with two fainter bands at R_f 0.38 and 0.25. Their polarity and absorption spectra were unaffected by dilute acid or alkali. There is evidence that they are breakdown products of diaponeurosporene and possess hydroxyl or carbonyl groups (19).

4,4′-Diaponeurosporenol. 4,4′-Diaponeurosporenol is a major xanthophyll in *Streptococcus faecium* (35, 38), and it was anticipated that it would be present in *S. aureus* also, as an intermediate if not a major component. Several specific searches of appropriate fractions from both the wild-type organism and several mutant strains failed to detect it. Authentic material for comparison was readily prepared by reduction of staphyloxanthin or diaponeurosporenoic acid with $LiAlH_4$.

Carotenoids in other strains of *S. aureus*. Pigment formation in *S. aureus* is known to vary in different strains, and it is also dependent on growth conditions. The results presented here were obtained with strain S41 grown under conditions which gave good yields of pigment. A survey of a number of orange-pigmented strains showed that they possessed the same carotenoid pattern as strain S41, with staphyloxanthin as the major component. A more detailed analysis of two strains, the neotype strain of *S. aureus* (NCTC 8532) and strain 209P (NCTC 7447), the strain used by Suzue (31) and by Taylor and Davies (Taylor and Davies, 4th Int. Symp. Carotenoids Abstr. Commun.) is presented in Table 7. An investigation of other pigment types and of the influence of growth conditions on pigmentation will be reported elsewhere.

TABLE 7. *Comparison of carotenoid content of three strains of S. aureus*

Carotenoid	Amt[a] (μg/g [dry wt])		
	S41[b]	8532	209P
4,4′-Diapophytoene	40	60	55
4,4′-Diapophytofluene	3	2	2
4,4′-Diapo-ζ-carotene	6	4	4
4,4′-Diaponeurosporene	12	8	8
4,4′-Diaponeurosporenal	tr		tr
4,4′-Diaponeurosporenoic acid	40	45	65
Glucosyl-diaponeurosporenoate	10	20	25
Staphyloxanthin	360	220	320
Hydroxy-400 compounds	10	5	8

[a] For growth conditions, see Table 2, footnote c.
[b] Strain.

DISCUSSION

Much of the earlier work on the chemical nature of the pigments of *S. aureus* is confused and contradictory. Some of the reasons for this are now clear. While it has been generally accepted that the pigments were carotenoids, it was assumed that, like most naturally occurring carotenoids, they possessed a C_{40} chain and that identification was only a matter of matching their electronic absorption spectra with the spectra of known carotenoids. Suzue et al. (33), however, presented clear evidence that the "bacterial phytoene" which they isolated from a strain of *S. aureus* possessed a C_{30} and not a C_{40} structure; our results now show that the whole series of carotenoid-like pigments in *S. aureus* belong to a C_{30} series, none having a C_{40} structure. This triterpenoid carotenoid series was first described and studied by Taylor and Davies (35, 37; Taylor and Davies, 4th Int. Symp. Carotenoids Abstr. Commun.). The compounds were isolated from a yellow-pigmented group D streptococcus (*Streptococcus faecium* UNH564P), and a review of C_{30} carotenoids by Davies (7) also includes information about some members of the series obtained from *S. aureus* 209P (partly based on unpublished data). Triterpenoid carotenoids cannot be distinguished from corresponding tetraterpenoids on the basis of their electronic absorption spectra, the spectra being very similar or identical, and conclusive evidence as to their molecular size must be obtained in other ways, such as measurement of retention times by GLC, measurement of R_f values in appropriate TLC systems, and determination of mass spectra.

A second unusual feature of staphylococcal pigments which has misled other workers is the instability of staphyloxanthin towards acid or alkali. The ester linkage between diaponeurosporenoic acid and glucose is readily split by dilute alkali, yielding the free acid or, in alkaline methanol, its methyl ester. Standard methods for extraction of carotenoids frequently employ alkaline methanol or, alternatively, employ a saponification step early in the purification procedure. Such treatment will rapidly convert staphyloxanthin to either diaponeurosporenoic acid or its methyl ester, which will then be isolated as apparently the main pigment component. Hammond and White in their study of *S. aureus* pigments (10) did consider the possibility that saponification may affect their product but concluded that it did not and that no esters or glycosides were present, since the products obtained had the same mobilities on alumina paper chromatography whether a saponification step was included or not.

Staphyloxanthin proved difficult to purify, being accompanied by glycolipid material in several chromatographic systems, and analyses of earlier samples of what was thought to be pure

material suggested that it contained glycerol as well as hexose and fatty acid. The most successful method of freeing it from glycolipid proved to be TLC on $AgNO_3$-impregnated silica gel.

Carotenoid glycosides have been found in several procaryotes, including *Streptococcus faecium* (35), cyanobacteria (11), and myxobacteria (14, 15), but staphyloxanthin is unusual among natural products in possessing not a glycosidic bond linking glucose to a hydroxy-carotenoid but a glucosyl ester bond linking glucose to a carotenoic acid. There is also evidence that, in addition to the main structure containing glucose, small amounts of isomers containing galactose or mannose are also present. A second glucosyl ester bond links glucose to a fatty acid, predominantly the C_{15} anteiso- acid 12-methyltetradecanoic acid, which is the major fatty acid found in other lipids of *S. aureus* (44); it is also accompanied by some of its C_{17} homolog, 14-methylhexadecanoic acid. A similar fatty acyl glucose linkage was found in the acylated carotenoid glycosides of *Nocardia kirovani* (39) and of some gliding bacteria, including *Stigmatella aurantiaca* (15) and *Herpetosiphon giganteus* (14), the acyl moieties here also consisting of mixtures of fatty acids. The position of attachment to glucose of the carotenoic acid (1-*O*-) and fatty acid (6-*O*-) were suggested by Moss (20) on the basis of nuclear magnetic resonance and other studies. Isostaphyloxanthin was never isolated in sufficient quantity for detailed structural studies, but its properties suggest that it is an isomer of staphyloxanthin, differing from it only in the position of attachment of the two acyl groups. Final confirmation of these structures may have to await the application of appropriate synthetic methods (22).

Triterpenoid carotenoids in several types of gram-positive cocci have been reported and may provide a useful taxonomic feature. However, there are several differences between those found in *S. aureus* and those found in *Streptococcus faecium.* The carotenes are identical and include both 4,4′-diapo-ζ-carotene and its unsymmetrical isomer 4,4′-diapo-7,8,11,12-tetrahydrolycopene and *cis* isomers of 4,4′-diaponeurosporene, which may be artifacts produced during isolation; Davies, however, reported only the first of these in *S. aureus* (7). In *Streptotoccus faecium*, the main xanthophylls are the 4-hydroxy- and 4-glucosyloxy- derivatives of 4,4′-diaponeurosporene, with a trace of the corresponding aldehyde; in *S. aureus*, 4,4′-diaponeurosporenoic acid, its glucosyl ester, staphyloxanthin, and a trace of aldehyde were all identified, but no hydroxy compound was found, nor have we been able to find evidence for a 4′-hydroxy- or a 4′-glucosyloxy- derivative of 4,4′-diaponeurosporenoic acid as postulated by Davies (7). The strain of *S. aureus* used for much of this work (S41) was different from that used by Taylor and Davies (209P), but we also examined their strain, the neotype strain NCTC 8532, and other orange-pigmented strains, and essentially the same carotenoid pattern was found for each. Other strains readily distinguished from the predominant orange type by the different color of their colonies also produced triterpenoid carotenoids but in different proportions or they lacked one or more members of the series. The carotenoid glycoside isolated from two yellow halophilic cocci of uncertain taxonomic position by Aasen et al. (1) appears to be a closely related triterpenoid carotenoid possessing a mannosyloxy- group at one end of the molecule and a carboxymethyl- group at the other, although the methyl ester may be an artifact of the saponification procedure used.

The evidence available at present is not sufficient to permit an unequivocal decision between two possible arrangements of the carbon chain of triterpenoid carotenoids (Fig. 5): the symmetrical 4,4′-diapo- structure proposed by Taylor and Davies (34) or the unsymmetrical 8′-apo- structure proposed by Aasen et al. (1). It is possible, but we consider it unlikely, that both forms may occur naturally. Evidence based on a comparison of samples of our staphylococcal carotenes with synthetic models led Moss to favor the unsymmetrical structure (20), but consideration of their biosynthetic origin favors the symmetrical one (7). 4,4′-Diapophytoene could be formed directly by condensation of two molecules of farnesyl pyrophosphate (C_{15} + C_{15}) analogous to phytoene formation (C_{20} + C_{20}). Formation of 8′-apophytoene, however, would require either the loss of C_{10} from a C_{40} chain or the condensation of two unequal units (C_{20} + C_{10}). The first mechanism would require a C_{40} intermediate, whereas in the second, unless the enzyme possessed very high specificity, some condensation products involving C_{15} would be expected, leading to C_{25} or C_{35} products. Neither in the work described here nor in the work with mutant strains (19) was there any evidence for C_{25}, C_{35}, or C_{40} products and, consequently, until more conclusive evidence is available, we propose to use the symmetrical structure.

The triterpenoid carotenoids of *S. aureus* described here possess structures which suggest they may constitute sequential steps in a pathway for the biosynthesis of staphyloxanthin. Details of this pathway and further evidence in support of it will be presented in the accompanying paper (19).

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LITERATURE CITED

1. **Aasen, A. J., G. W. Francis, and S. Liaaen-Jensen.** 1969. Bacterial carotenoids. XXIX. The carotenoids of two yellow halophilic cocci—including a new glycosidic methyl apolycopenoate. Acta Chem. Scand. **23**:2605–2615.
2. **Aasen, A. J., and S. Liaaen-Jensen.** 1966. Carotenoids of flexibacteria. III. The structures of flexixanthin and deoxyflexixanthin. Acta Chem. Scand. **20**:1970–1988.
3. **Allegra, G., R. Niutta, and G. Giuffrida.** 1954. Studi sulla carotenogenesi e sul ruolo dei carotenoidi nella *Staphylococcus aureus*. I. Azione del glucosio sulla cromogenesi dello *Staphylococcus aureus* (stipite Oxford). Riv. Ist. Sieroter. Ital. **29**:263–269.
4. **Baird-Parker, A. C.** 1972. Classification and identification of staphylococci and their resistance to physical agents, p. 1–20. *In* J. O. Cohen (ed.), The staphylococci. John Wiley & Sons, Inc., New York.
5. **Chapman, D.** 1964. Structure of lipids, p. 133–159. Methuen and Co. Ltd., London.
6. **Chargaff, E.** 1933. Sur les caroténoïdes des bactéries. C.R. Acad. Sci. **197**:946–948.
7. **Davies, B. H.** 1977. C_{30} carotenoids, p. 51–100. *In* T. W. Goodwin (ed.), International review of biochemistry. Biochemistry of lipids II, vol. 14. University Park Press, Baltimore.
8. **Davies, B. H., and R. F. Taylor.** 1976. Carotenoid biosynthesis—the early steps. Pure Appl. Chem. **47**:211–221.
9. **Douglas, R. J., and J. T. Parisi.** 1966. Chromogenesis by variants of *Staphylococcus aureus*. J. Bacteriol. **92**: 1578–1579.
10. **Hammond, R. K., and D. C. White.** 1970. Carotenoid formation by *Staphylococcus aureus*. J. Bacteriol. **103**: 191–198.
11. **Hertzberg, S., and S. Liaaen-Jensen.** 1969. The structure of myxoxanthophyll. Phytochemistry **8**:1259–1280.
12. **Hertzberg, S., S. Liaaen-Jensen, C. R. Enzell, and G. W. Francis.** 1969. Animal carotenoids. 3. The carotenoids of *Actinia equina*—structure determination of actinioerythrin and violerythrin. Acta Chem. Scand. **23**: 3290–3312.
13. **Klebe, J. F., H. Finkbeiner, and D. M. White.** 1966. Silylations with bis(trimethylsilyl)acetamide, a highly reactive silyl donor. J. Am. Chem. Soc. **88**:3390–3396.
14. **Kleinig, H., and H. Reichenbach.** 1977. Carotenoid glucosides and menaquinones from the gliding bacterium *Herpetosiphon giganteus* Hp a2. Arch. Microbiol. **112**:307–310.
15. **Kleinig, H., H. Reichenbach, and H. Achenbach.** 1970. Carotenoid pigments of *Stigmatella aurantiaca* (Myxobacterales). II. Acylated carotenoid glucosides. Arch. Mikrobiol. **74**:223–234.
16. **Krebs, K. G., D. Heusser, and W. Wimmer.** 1969. Spray reagents, p. 854–905. *In* E. Stahl (ed.), Thin layer chromatography, 2nd ed. Springer-Verlag, New York.
17. **Kushwaha, S. C., E. L. Pugh, J. K. G. Kramer, and M. Kates.** 1972. Isolation and identification of dehydrosqualene and C_{40}-carotenoid pigments in *Halobacterium cutirubrum*. Biochim. Biophys. Acta **260**:492–506.
18. **Liaaen-Jensen, S.** 1971. Isolation, reactions, p. 61–188. *In* O. Isler (ed.), Carotenoids. Birkhaüser-Verlag, Basel.
19. **Marshall, J. H., and G. J. Wilmoth.** 1981. Proposed pathway of triterpenoid carotenoid biosynthesis in *Staphylococcus aureus*: evidence from a study of mutants. J. Bacteriol. **147**:914–919.
20. **Moss, G. P.** 1979. Physico-chemical and synthetic studies on carotenoids. Pure Appl. Chem. **51**:507–514.
21. **Petracek, F. J., and L. Zechmeister.** 1956. Determination of partition coefficients of carotenoids as a tool in pigment analysis. Anal. Chem. **28**:1484–1485.
22. **Pfander, H.** 1979. Synthesis of carotenoid glycosylesters and other carotenoids. Pure Appl. Chem. **51**:565–580.
23. **Pfander, H., and F. Wittwer.** 1975. Carotenoid-Glykoside. Untersuchungen zur Carotinoid-Zusammensetzung im Safran. Helv. Chim. Acta. **58**:1608–1620.
24. **Porter, J. W., and D. G. Anderson.** 1962. The biosynthesis of carotenes. Arch. Biochem. Biophys. **97**:520–528.
25. **Porter, J. W., and R. E. Lincoln.** 1950. I. Lycopersicon selections containing a high content of carotene and colorless polyenes. II. The mechanism of carotene biosynthesis. Arch. Biochem. Biophys. **27**:390–403.
26. **Rosenbach, F. J.** 1884. Mikroorganismen bei den Wundinfektionskrankheiten des Menschen. J. F. Bergman, Wiesbaden.
27. **Schindler, J.** 1972. Pigment production of *Staphylococcus aureus* PS 80 and its mutants. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. Reihe A **221**:157–165.
28. **Schleifer, K. H., and M. Kocur.** 1973. Classification of staphylococci based on chemical and biochemical properties. Arch. Mikrobiol. **93**:65–85.
29. **Sobin, B., and G. L. Stahly.** 1942. The isolation and absorption spectrum maximum of bacterial carotenoid pigments. J. Bacteriol. **44**:265–276.
30. **Steuer, W.** 1956. Untersuchungen über die Pigmentbildung von *Micrococcus pyogenes*. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. Reihe A **167**:210–216.
31. **Suzue, G.** 1959. Studies on carotenogenesis by *Micrococcus pyogenes* var. *aureus*. J. Biochem. (Tokyo) **46**: 1497–1504.
32. **Suzue, G., K. Tsukada, and S. Tanaka.** 1967. A new triterpenoid from a mutant of *Staphylococcus aureus*. Biochim. Biophys. Acta **144**:186–188.
33. **Suzue, G., K. Tsukada, and S. Tanaka.** 1968. Occurrence of dehydrosqualene (C_{30} phytoene) in *Staphylococcus aureus*. Biochim. Biophys. Acta **164**:88–93.
34. **Taylor, R. F., and B. H. Davies.** 1974. Triterpenoid carotenoids and related lipids. The triterpenoid carotenes of *Streptococcus faecium* UNH 564P. Biochem J. **139**:751–760.
35. **Taylor, R. F., and B. H. Davies.** 1974. Triterpenoid carotenoids and related lipids. Triterpenoid monohydroxy- and monoglucosyloxy-carotenoids from *Streptococcus faecium* UNH 564P. Biochem. J. **139**:761–769.
36. **Taylor, R. F., and B. H. Davies.** 1975. Gas-liquid chromatography of carotenoids and other terpenoids. J. Chromatogr. **103**:327–340.
37. **Taylor, R. F., and B. H. Davies.** 1976. Triterpenoid carotenoids and related lipids. Triterpenoid carotenoid aldehydes from *Streptococcus faecium* UNH 564P. Biochem. J. **153**:233–239.
38. **Taylor, R. F., and B. H. Davies.** 1976. The influence of culture conditions on carotenogenesis in *Streptococcus faecium* UNH 564P. J. Gen. Microbiol. **92**:325–334.
39. **Vacheron, M. J., N. Arpin, and G. Michel.** 1970. Isolement d'esters de phlei-xanthophylle de *Nocardia kirovani*. C.R. Acad. Sci. Sér C **271**:881–884.
40. **Vaughan, D. H., W. S. Riggsby, and J. O. Mundt.** 1979. Deoxyribonucleic acid relatedness of strains of yellow-pigmented, group D streptococci. Int. J. Syst. Bacteriol. **29**:204–212.
41. **Waldi, D.** 1965. Dünnschicht-Chromatographie einiger Zucker and Zuckeralkohole. J. Chromatogr. **18**:417–418.

42. **Walter, W. M., and A. E. Purcell.** 1966. Elimination of color quench in liquid scintillation counting of ^{14}C-carotenoids. Anal. Biochem. **16**:466–473.
43. **Weeks, O. B., and R. J. Garner.** 1967. Biosynthesis of carotenoids in *Flavobacterium dehydrogenans* Arnaudi. Arch. Biochem. Biophys. **121**:35–49.
44. **White, D. C., and F. E. Frerman.** 1967. Extraction, characterization, and cellular localization of the lipids of *Staphylococcus aureus*. J. Bacteriol. **94**:1854–1867.
45. **Willis, A. T., S. I. Jacobs, and G. M. Goodburn.** 1964. Pigment production, enzymatic activity and antibiotic sensitivity of staphylococci. Sub-division of the pathogenic group. J. Pathol. Bacteriol. **87**:157–167.
46. **Willis, A. T., and G. C. Turner.** 1962. Staphylococcal lipolysis and pigmentation. J. Pathol. Bacteriol. **84**:337–347.
47. **Wood, R., and F. Snyder.** 1965. Modified silver-ion thin-layer chromatography. J. Am. Oil Chem. Soc. **43**:53–54.

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Structure and Biosynthesis of Staphyloxanthin from *Staphylococcus aureus*

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Structure and Biosynthesis of Staphyloxanthin from *Staphylococcus aureus**

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Most *Staphylococcus aureus* strains produce the orange carotenoid staphyloxanthin. The staphyloxanthin biosynthesis genes are organized in an operon, *crtOPQMN*, with a σ^B-dependent promoter upstream of *crtO* and a termination region downstream of *crtN*. The functions of the five encoded enzymes were predicted on the basis of their sequence similarity to known enzymes and by product analysis of gene deletion mutants. The first step in staphyloxanthin biosynthesis is the head-to-head condensation of two molecules of farnesyl diphosphate to form dehydrosqualene (4,4′-diapophytoene), catalyzed by the dehydrosqualene synthase CrtM. The dehydrosqualene desaturase CrtN dehydrogenates dehydrosqualene to form the yellow, main intermediate 4,4′-diaponeurosporene. CrtP, very likely a mixed function oxidase, oxidizes the terminal methyl group of 4,4′-diaponeurosporene to form 4,4′-diaponeurosporenic acid. CrtQ, a glycosyltransferase, esterifies glucose at the C_1'' position with the carboxyl group of 4,4′-diaponeurosporenic acid to yield glycosyl 4,4′-diaponeurosporenoate; this compound was the major product in the clone expressing *crtPQMN*. In the final step, the acyltransferase CrtO esterifies glucose at the C_6'' position with the carboxyl group of 12-methyltetradecanoic acid to yield staphyloxanthin. Staphyloxanthin overexpressed in *Staphylococcus carnosus* (pTX-*crtOPQMN*) and purified was analyzed by high pressure liquid chromatography-mass spectroscopy and NMR spectroscopy. Staphyloxanthin was identified as β-D-glucopyranosyl 1-*O*-(4,4′-diaponeurosporen-4-oate)-6-*O*-(12-methyltetradecanoate).

The species epithet of *Staphylococcus aureus* reflects the color of its colonies (L. *aureus*: golden) and distinguish this species from *Staphylococcus epidermidis* (formerly *Staphylococcus albus*) (1). The orange pigmentation of *S. aureus* had been used as a species character until colorless strains were observed (2). The pigment name staphyloxanthin was first mentioned by Marshall and Rodwell (33). In pioneering work, Marshall and Wilmoth (3) isolated the pigments from *S. aureus* and chemically analyzed 17 intermediary products, identifying them as triterpenoid carotenoids possessing a C_{30} chain instead of the C_{40} carotenoid structure found in most other organisms (4). The main pigment, staphyl oxanthin, was identified as α-D-glucopyranosyl 1-*O*-(4,4′-diaponeurosporen-4-oate)-6-*O*-(12-methyltetradecanoate), in which glucose is esterified with both a triterpenoid carotenoid carboxylic acid and a C_{15} fatty acid.

In previous work, we cloned the genes for staphyloxanthin biosynthesis from *S. aureus* and analyzed the function of two enzymes involved in the pathway (5). The biosynthesis of the pigment starts with the head-to-head condensation of two farnesyl diphosphate molecules, catalyzed by the dehydrosqualene synthase CrtM, to yield 4,4′-diapophytoene (dehydrosqualene). Dehydrosqualene desaturase, CrtN, catalyzes the formation of the first deep yellow-colored carotenoid intermediate product, 4,4′-diaponeurosporene, which is formed via successive dehydrogenation reactions (5).

Here, we analyzed the complete staphyloxanthin biosynthesis operon *crtOPQMN*. We postulated the function of the encoded proteins based on product analysis of *crt* mutants and sequence similarity comparisons. Staphyloxanthin was purified, and its structure was determined by NMR spectroscopy.

MATERIALS AND METHODS

Growth Media, DNA Manipulations, and Transformation—Standard procedures for DNA manipulations, plasmid DNA isolation, transformation of *Escherichia coli*, and preparation of liquid media and agar plates for bacterial growth were followed. Plasmid DNA was introduced into *Staphylococcus carnosus* strain TM300 and *S. aureus* strain Newman by protoplast transformation (6). *S. carnosus* and *S. aureus* were grown in tryptic soy broth medium (Invitrogen) or LB medium (1% tryptone, 0.5% yeast extract, and 0.5% NaCl). The plasmid vectors pCA44 (7, 8) and the xylose-inducible pTX15 (9, 10) were used; for induction in the latter case, xylose was added to the medium to a final concentration of 0.5%. PCR was carried out with Vent® polymerase (New England Biolabs) or Expand™ Long Template PCR System (Roche Applied Science). The staphyloxanthin biosynthesis genes upstream of *crtM* were sequenced via primer walking using a LI-COR DNA sequencer Long Reader 4200 (Lincoln Corporation, Lincoln, NE). Sequences were analyzed using MacDNASIS Pro software (Hitachi Software Engineering, San Bruno, CA).

Primers used for PCR, primer extension, and PCR-digoxigenin labeling. The following primers were used to amplify the *crt* genes: 1-N/R, TTTGGATCCTTGCTTCTGCAGGTCATCCGG; L3SstI, TTTGAGCTCTTGTATAACGCCCATCAAGGTACG; crtP/R, TTTGGATCCAGATTAACGCAGTTACTACGC; crtQ/R, TTTGGATCCTTATCGTTCTAATCGTGGTGC; MN/R, TTTGGATCCTTCTGCATCGAAGGGTCGGCC; and NN/R, TTTGGATCCTTCTTGGACGAAGTTGAGACAGGC. The following primers were used to amplify the 515-bp *crt* promoter fragment: PR, TTGGATCCTTGTGACTACAACTGCAGCGCC; and PL, TTTGAGCTCTTCACTCTCAATCATACTGAC. For the PCR-digoxigenin labeling, the following primers were used: crtM/R2, TTTGGATCCTTAACAAACGCATCGTTATGG; crtM/L2, TTTGAGCTCTTCATGAAACAACTTTGCC; IK 15, ATGGTCATCTTGTTGCCCC; and IK 16, GAATACAGATGCACAGG.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) X97985.
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The nucleotides underlined in the primers indicate a BamHI or SstI restriction site.

Growth Conditions and Pigment Extraction—Staphyloxanthin and intermediate carotenoids were isolated from recombinant *S. carnosus* clones. Cells were grown at 37 °C for 24 h in 0.5 liters of tryptic soy broth supplemented with 0.5% xylose. Cultures were centrifuged, and the cell pellets were either used immediately or stored at −70 °C; at this temperature, the carotenoids were stable for several months. Pigments were extracted by resuspending the cell pellet in 10 ml of ethanol and incubating for 20 min at 40 °C. After centrifugation, the supernatant containing the pigments was concentrated to small volumes *in vacuo* and then extracted with ethyl acetate/1.7 M aqueous NaCl (1:1, v/v). The colored ethyl acetate extract was dried with anhydrous Na_2SO_4, and the solvent was removed *in vacuo* (crude extract). The residue was dissolved in ethyl acetate and subjected to silica gel 60 (1.5–4.0 μm, Merck) column chromatography. The colored fractions were eluted with ethyl acetate, and the individual fractions were evaporated to dryness. Because of the light sensitivity of the pigments, all further purification steps were carried out in the dark.

Thin-layer Chromatography (TLC) and Spectral Analysis—Pigment crude extracts were separated on RP-18 F_{254S} plates (Merck) with methanol-acetonitrile (1:1, v/v). The pigment bands were scratched off the preparative TLC plates and dissolved in ethyl acetate, and the absorption spectra were recorded (Uvikon 940, Kontron).

HPLC—Carotenoid extracts were analyzed by HPLC[2] on an HP1100 HPLC system (Agilent Technologies, Waldbronn, Germany) with a 250 × 4.6 mm ProntoSil C30 stainless steel column (particle size 3 μm, average pore diameter 200 Å; Bischoff, Leonberg, Germany) and UV light detection at 450 nm. The compounds (50-μl injection sample) were separated with an acetone/water gradient (0 min, 90% acetone; 0–20 min, linear gradient to 100% acetone; 21–30 min, 100% acetone) at a flow rate of 1 ml/min.

HPLC-MS—Mass spectroscopy was performed on a Brucker Esquire 3000plus ion-trap mass spectrometer (Bruker Daltonik, Bremen, Germany) equipped with an atmospheric pressure chemical ionization ion source. The mass spectra were recorded in the positive ion mode in the mass range from *m/z* 300 to 1000. The voltage of the corona needle was optimized, resulting in a current of 4–8 μA. Nitrogen was the drying and carrier gas (300 °C). The temperature of the ionization chamber was set to 300 °C.

GC-MS—The GC-MS measurements were performed on a HP 6890/5970 GC-MS system. For the sugar verification, 0.6 N HCl in methanol (200 μl) and 50 μl of methylacetate was added to the compound. The solution was stored for 16 h at 70 °C. Then dichloromethane was added, and the solvent was removed under a nitrogen stream at room temperature. 30 μl of absolute pyridine and bis(trimethylsilyl)trifluoracetamide was added and heated for one h at 60 °C. The GC separation was performed on a DB-5 column. To verify the result, the measurement was repeated with an added glucose standard.

NMR Spectroscopy—The structure of staphyloxanthin was determined on a computer-controlled Bruker 700 MHz UltraShield Spectrometer (magnetic field strength 16.44 Tesla, proton resonance 700.13 MHz) at Bruker Biospin AG (Faellanden, Switzerland) using preparative amounts purified from *S. carnosus* (pTX*crtOPQMN*). A $^1H\{^{13}C/^{15}N\}$ cryoprobe with a Z gradient (700 MHz, 5 mm TXI H-C/N; Bruker Biospin AG) was used. The data were analyzed with XWINNMR 3.0 software (Bruker Daltonik) in our laboratory. All resonances for the structure elucidation of staphyloxanthin were unambiguously assigned; the results from one-dimensional ^1H and ^{13}C NMR spectra and from two-dimensional NMR spectra ($^1H,^1H$-COSY, TOCSY, HSQC, HMBC, NOESY, ROESY) were combined.

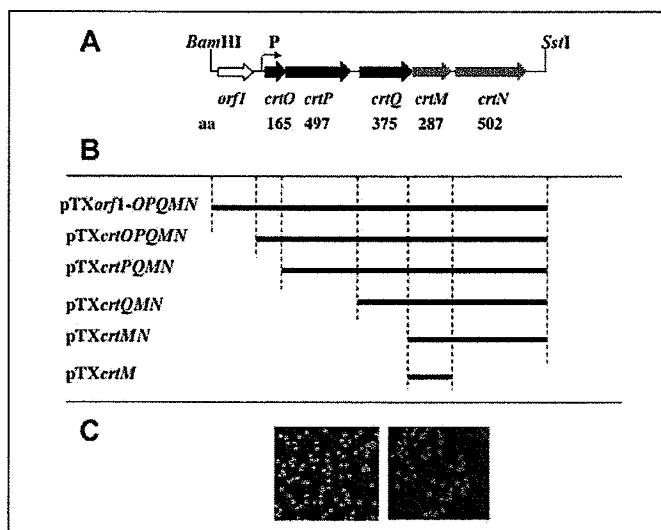

FIGURE 1. ***crt* operon and its expression in *S. carnosus*.** *A*, organization of the *crt* genes in the *S. aureus* Newman genome (sequence accession X97985, submitted in 1996). The same organization is also found in the published genome of *S. aureus* N315: *crtO* (SA2352), *crtP* (SA2351), *crtQ* (SA2350), *crtM* (SA2349), and *crtN* (SA2348). *B*, construction of truncated *crt* expression plasmids. *C*, colonies of *S. carnosus* wild-type (*left*) and *S. carnosus* (pTX*crtOPQMN*) expressing staphyloxanthin (*right*).

RESULTS

The staphyloxanthin biosynthesis operon *crt*. We cloned a 12-kb DNA fragment from *S. aureus* strain Newman (ATCC 25904) yielding plasmid pOC1. When unpigmented *S. carnosus* was transformed with pOC1, the colonies of the transformants produced staphyloxanthin and were orange, which suggested that all genes necessary for the synthesis of staphyloxanthin were present on pOC1.

The function of two biosynthesis genes had been analyzed earlier; *crtM* encodes dehydrosqualene synthase, and *crtN* encodes dehydrosqualene desaturase (5). In the current study, the nucleotide sequence revealed an operon composed of five genes (Fig. 1*A*). Following our previous nomenclature of *crtM* and *crtN*, we named the upstream genes *crtO*, *crtP*, and *crtQ* (operon *crtOPQMN*). We cloned the *crt* genes in the xylose-inducible expression vector pTX15 by deleting stepwise gene by gene from the 5′-end (Fig. 1*B*). Staphyloxanthin expression studies showed that all five genes are necessary for staphyloxanthin biosynthesis. The nucleotide sequences of the staphyloxanthin biosynthesis genes of *S. aureus* strain Newman and *S. aureus* N315 (11) are almost identical. When expression of *crtOPQMN* was induced in *S. carnosus*, colonies were strongly orange pigmented (Fig. 1*C*).

A functional *sigB* operon is necessary for the synthesis of staphyloxanthin in *S. aureus* (12). The identification in the current study of a σ^B-dependent promoter upstream of *crtO* explains this requirement. In earlier studies, we were uncertain whether the open reading frame orf1 lying upstream of the *sigB* promoter region is involved in staphyloxanthin biosynthesis. Here we showed that this gene is not involved in staphyloxanthin biosynthesis because its deletion had no effect on staphyloxanthin production. Furthermore, the predicted protein product of orf1 shows sequence similarity to the staphylococcal secretory antigen (13).

In the published *S. aureus* genome sequences, the *crtOPQNM* genes are highly conserved, have the same gene organization, and appear to be located at the same genomic site. Data base searches with CrtOPQ proteins revealed only for the CrtP and CrtQ hints as to their function.

[2] The abbreviations used are: HPLC, high pressure liquid chromatography; MS, mass spectroscopy; GC, gas chromatography; HMG, 3-hydroxy-3-methylglutaryl.

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CrtO (20.3 kDa) has no sequence similarities to any other carotenoid biosynthesis proteins known. The highest sequence similarity (identity: 34%; similarity: 59%) is with a protein from *Oceanobacillus iheyensis* HTE831, an alkaliphilic and extremely halotolerant *Bacillus*-related species isolated from deep sea sediment (14). We propose that CrtO is an acyltransferase that catalyzes the last step in staphyloxanthin biosynthesis, namely the acetylation of glucosyl-4,4′-diaponeurosporenoate.

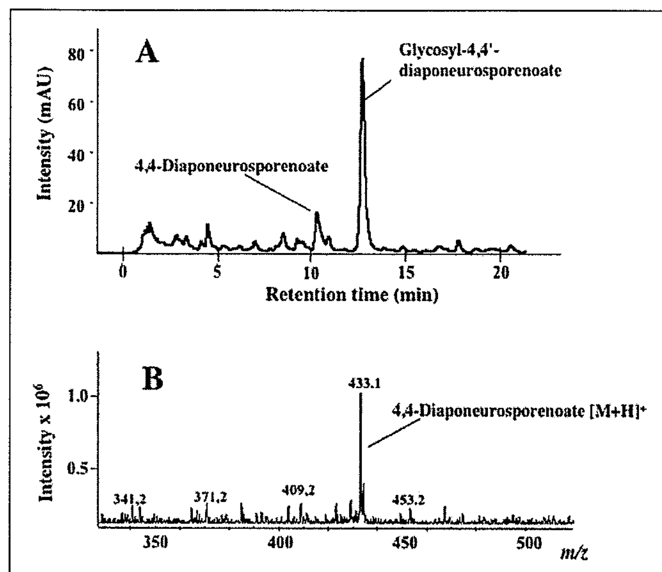

FIGURE 2. **Analysis of the major product of *S. carnosus* (pTX*crtPQMN*).** *A*, HPLC-UV analysis revealed glycosyl-4,4′-diaponeurosporenoate (absorption maxima, 460 and 483 nm) as a major peak and 4,4′-diaponeurosporenoate (absorption maxima, 455 and 483 nm) as a minor peak. *B*, mass analysis of glycosyl-4,4′-diaponeurosporenoate yielded the unglycosylated form because of the instability of the glycosidic bond to ionization. Note, glucose was separately verified by GC-MS.

CrtP (50.8 kDa) shows high sequence similarity to phytoene dehydrogenase of *Heliobacillus mobilis* (identity, 29%; similarity, 93%) (15), *Myxococcus xanthus* (identity, 29%; similarity, 50%) (16), *Pantoea agglomerans* (formerly *Erwinia herbicola*; identity, 27%; similarity, 48%) (17), and *Streptomyces griseus* (identity, 28%; similarity, 61%) (18). Interestingly, CrtP shows some similarity to the dehydrosqualene desaturase CrtN of *S. aureus* (identity, 24%; similarity, 48%) (5). CrtP very likely catalyzes the oxidation of 4,4′-diaponeurosporene to 4,4′-diaponeurosporenoate and would therefore be regarded as a diaponeurosporene oxidase.

CrtQ (42.5 kDa) shows sequence similarities to galactosyltransferase and glycosyltransferase of *Prochlorococcus marinus* (identity, 26%; similarity, 44%) (19) and *Chlorobium tepidum* (identity, 25%; similarity, 43%) (20) and to glycosyltransferases involved in cell wall biogenesis in *Magnetospirillum magnetotacticum* (identity, 26%; similarity, 42%) (accession number: NZ_AAAP01002898). The highest similarity was again found with a hypothetical conserved protein of *O. iheyensis* HTE831 (identity, 36%; similarity, 57%) (14). Interestingly, CrtQ also has similarity to the processive glycosyltransferase IcaA of *S. aureus* (identity: 23%; similarity: 41%) and *S. epidermidis* (identity: 22%; similarity: 41%) (21–23). Five strictly conserved amino acids are involved in the catalytic function of processive glycosyltransferases. In a β,β,β-glycosyltransferase model, the catalytic amino acids are organized in two domains. Domain D1 contains one amino acid, and domain D2 contains four amino acids (24). Non-processive glycosyltransferases (transferases that transfer only one monosaccharide) have only the one con-

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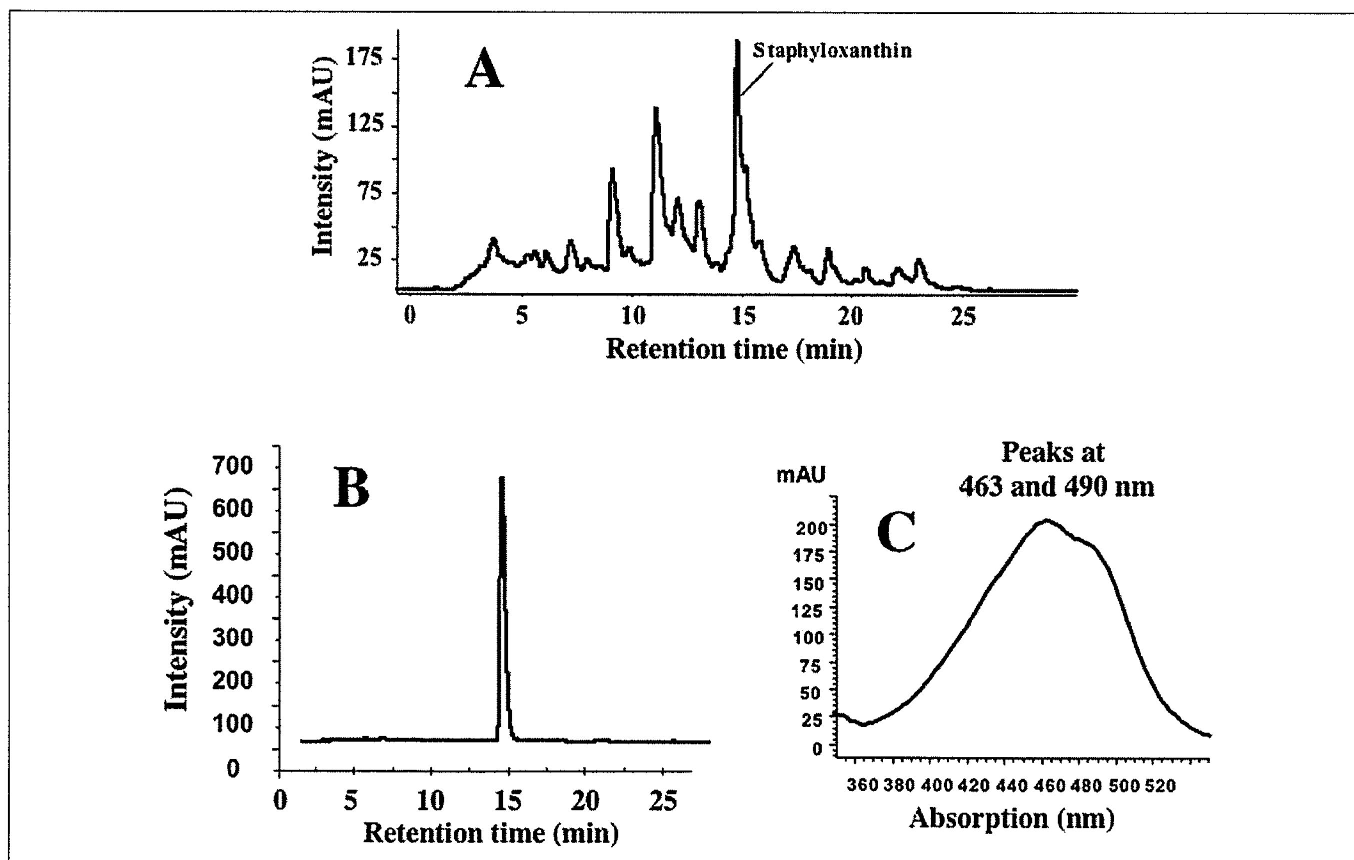

FIGURE 3. **HPLC analysis.** *A*, HPLC-UV analysis of a crude extract of *S. carnosus* (pTX*crtOPQMN*) separated on a C_{30} column. *B*, HPLC-UV analysis of purified staphyloxanthin used for NMR analysis. *C*, Absorption spectrum of purified staphyloxanthin. Note the peaks at 463 and 490 nm.

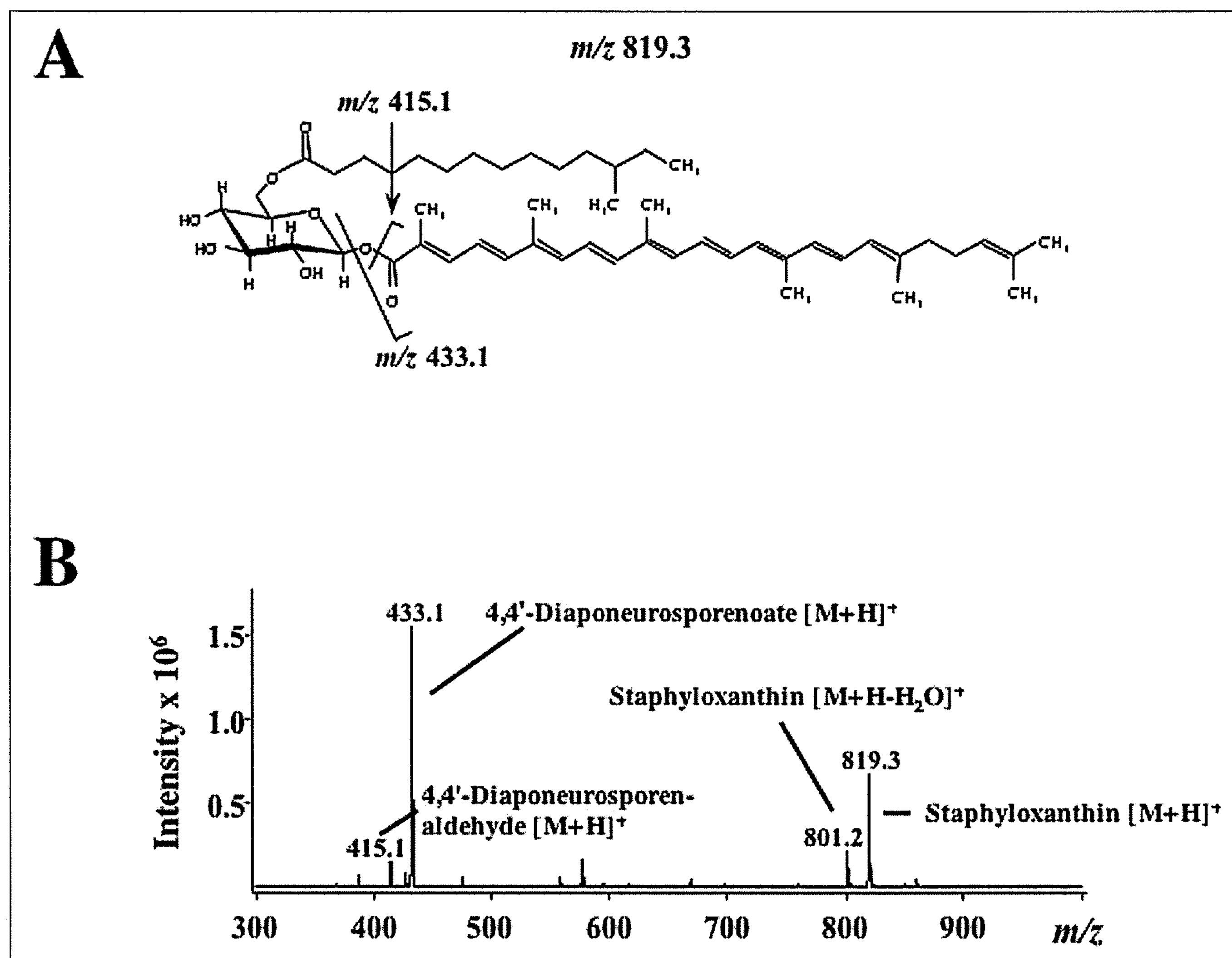

FIGURE 4. **NMR structure and mass analysis.** *A*, structure of staphyloxanthin elucidated from NMR spectroscopy analysis; the fragments obtained after ionization and the corresponding masses are indicated. *B*, NMR mass spectra. Identified compounds, ionizations, and masses are indicated.

served amino acid in domain D1. The alignment of the CrtQ and processive glycosyltransferase IcaA sequences shows that CrtQ has only the conserved amino acid in domain D1; no sequence similarities to the strictly conserved amino acids of domain D2 were detected. Therefore, CrtQ appears to be a member of the non-processive glycosyltransferase family and very likely catalyzes the glycosylation of 4,4′-diaponeurosporenoate. To investigate the function of carotenoid biosynthesis genes and the metabolic products, subsets of the *crt* genes were cloned into the xylose-inducible expression vector pTX15 (25) behind the *xylA* promoter, creating plasmids pTX-*orf1OPQMN*, pTX*crtOPQMN*, pTX-*crtPQMN*, pTX*crtQMN*, pTX*crtMN*, and pTX*crtM* (Fig. 1*B*).

The recombinant *S. carnosus* strains were cultivated for 24 h, and expression of the genes was induced with 1% xylose. The carotenoids were extracted and separated by preparative TLC, and the absorption spectrum of each individual pigment band was recorded. From the data obtained, it was largely possible to assign the genes to the steps of the staphyloxanthin biosynthesis pathway. As expected, expression of *crtM* did not lead to pigment production because the dehydrosqualene synthase CrtM converts farnesyl pyrophosphate to dehydrosqualene, which is colorless. Expression of *crtM* and *crtN* leads to the formation of the first yellow-colored C_{30} carotenoid, 4,4′-diaponeurosporene (absorption maxima: 415, 438, and 468 nm) (5). In *S. carnosus* (pTX-*crtQMN*), 4,4′-diaponeurosporene was again the major carotenoid found; the TLC carotenoid patterns derived from membrane extracts of clones containing either pTX*crtMN* or pTX-*crtQMN* did not differ, which can be explained by CrtQ not catalyzing the subsequent reaction.

Our data indicate that the oxidation of the terminal methyl side group of 4,4′-diaponeurosporene to 4,4′-diaponeurosporenoate is the next biosynthetic step and that this reaction is catalyzed by CrtP. CrtP is therefore a diaponeurosporene oxidase that converts 4,4′-diaponeurosporene very likely via an aldehyde, 4,4′-diaponeurosporenal (26) to the carboxylic acid carotenoid, 4,4′-diaponeurosporenoate.

The next proposed step, the glycosylation of 4,4′-diaponeurosporenoate to form glycosyl-4,4′-diaponeurosporenoate, is very likely catalyzed by CrtQ, which shows sequence similarity to glycosyltransferases. Indeed, we found that *S. carnosus* (pTX*crtPQMN*) produced as a major product glycosyl-4,4′-diaponeurosporenoate (absorption maxima: 460 and 483 nm) and to a minor extent 4,4′-diaponeurosporenoate (absorption maxima: 401, 422, and 444 nm) (Fig. 2*A*). To prove that the major peak represents glycosyl-4,4′-diaponeurosporenoate the corresponding carotenoid was purified and subjected to both MS and sugar analysis. The MS analysis yielded a mass of 433.1 that corresponds to 4,4′-diaponeurosporenoate $[M+H]^+$ (Fig. 2*B*). The reason why we did not see the glycosylated form was because of the high instability of the glycosidic bond to ionization. Therefore, we verified the sugar by GC-MS. The purified carotenoid was subjected to acid hydrolysis as

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TABLE ONE

Identified carotenoids in various *S. carnosus* (pTX*crt*) clones

Clones	Major products	Mass analysis	Absorption maxima
		m/z	*nm*
pTX*crtOPQMN*	Staphyloxanthin $[M+H]^+$	819.3	463, 490
	Staphyloxanthin $[M+H\text{-}H_2O]^+$	801.2	
	4,4-Diaponeurosporenoate $[M+H]^+$	433.1	455, 483
	4,4′-Diaponeurosporene-aldehyde $[M+H]^+$	415.1	
pTX*crtPQMN*	Glycosyl-4,4′-diaponeurosporenoate		460, 483
	4,4-Diaponeurosporenoate $[M+H]^+$	433.1	455, 483
pTX*crtQMN*	4,4′-Diaponeurosporene		415,438,468
pTX*crtMN*	4,4′-Diaponeurosporene		415,438,468
pTX*crtM*	No carotenoid produced		

described in Materials and Methods, and by GC separation glucose could be unambiguously identified. These results clearly indicate that CrtQ is the glycosyltransferase.

The last step in the pathway is the esterification of the glycosyl residue with a fatty acid. Only in clones containing pTX*crtOPQMN*, which carries all five *crt* genes, was staphyloxanthin produced. Although CrtO shows no sequence similarity to known enzymes, it must catalyze the last step in staphyloxanthin biosynthesis, namely the acylation of glycosyl-4,4′-diaponeurosporenoate. Structure analysis indicates that it is a glycosyl-C_6''-*O*-acyltransferase.

NMR Structure of Staphyloxanthin—The main carotenoid in the crude extract of *S. carnosus* (pTX*crtOPQMN*) was staphyloxanthin, as shown in the HPLC profile (Fig. 3*A*). The carotenoid was purified further by silica gel chromatography, followed by preparative HPLC, yielding a final compound with a high degree of purity (Fig. 3*B*) and an absorption spectrum with peaks at 463 and 490 nm, characteristic for staphyloxanthin (Fig. 3*C*). The NMR spectrum identified staphyloxanthin as a β-D-glucopyranosyl 1-*O*-(4,4′-diaponeurosporen-4-oate)-6-*O*-(12-methyltetradecanoate). The central core of the structure is glucose-esterified at position C_1'' with the carotenoid 4,4′-diaponeurosporenic acid and at position C_6'' with the C_{15} fatty acid 12-methyltetradecanoic acid (Fig. 4*A*). Our NMR structure of staphyloxanthin essentially confirmed the structure determined earlier in an excellent study by Marshall and Wilmoth (3) mainly by chemical methods. The only difference found is the configuration of glucose. In nature, D-sugars in glycosides are normally β-glycosidically linked, whereas l-sugars are α-glycosidically linked. Moss (27) proposed α-D-glucose as the glycoside of staphyloxanthin. Here we show that the core is a β-D-glucopyranose.

Characteristic fragments in the mass spectrum were obtained after ionization (Fig. 4*B*). The C_1'' glycosidic binding appears to be quite unstable to ionization because the peak at *m/z* 433.1 (4,4′-diaponeurosporenoate $[M+H]^+$) is much higher than the peak at *m/z* 819.3 (staphyloxanthin $[M+H]^+$). The mass spectrum also revealed peaks for staphyloxanthin $[M+H\text{-}H_2O]^+$ (*m/z* 801.2) and 4,4′-diaponeurosporenaldehyde [M] (*m/z* 415.1). A summary of some characteristics of the identified carotenoids in the various *S. carnosus* (pTX-) clones is shown in TABLE ONE.

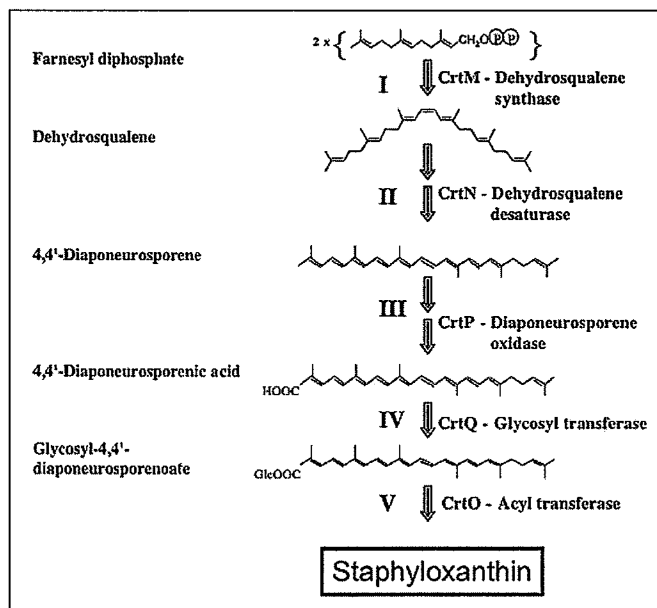

FIGURE 5. **Proposed staphyloxanthin biosynthesis pathway.**

DISCUSSION

We confirmed and extended the pathway proposed by Marshall and Wilmoth (26) by allocating the genes of the *crt* operon involved in staphyloxanthin biosynthesis (Fig. 5). Five genes and enzymatic reactions are involved: 1) condensation of two molecules of farnesyl diphosphate to form dehydrosqualene catalyzed by the dehydrosqualene synthase CrtM; 2) stepwise oxidation of dehydrosqualene to 4,4′-diaponeurosporene catalyzed by the dehydrosqualene desaturase CrtN; 3) oxidation of the terminal methyl group of 4,4′-diaponeurosporene to form 4,4′-diaponeurosporenic acid, catalyzed by CrtP, which is probably a mixed function oxidase; 4) esterification of glucose at the C_1'' position with the carboxyl group of 4,4′-diaponeurosporenic acid to yield glycosyl-4,4′-diaponeurosporenoate, catalyzed by the glycosyltransferase CrtQ; and 5) finally esterification of glucose at the C_6'' position with the carboxyl group of 12-methyltetradecanoic acid to yield staphyloxanthin, catalyzed by the acyltransferase CrtO.

The biosynthesis of staphyloxanthin starts with farnesyl diphosphate, which is involved in isoprenoid metabolism. Isoprenoids, derived from isopentenyl diphosphate and its isomer dimethylallyl diphosphate are essential for survival in most organisms. Two pathways for the synthesis of isopentenyl diphosphate are known: the mevalonate pathway and the glyceraldehyde 3-phosphate-pyruvate pathway. Genomic analyses have revealed that staphylococci, streptococci, enterococci, and Archaea possess the enzymes of the mevalonate pathway but not of the glyceraldehyde 3-phosphate-pyruvate pathway, whereas *Bacillus subtilis* and most Gram-negative bacteria studied possess only components of the glyceraldehyde 3-phosphate-pyruvate pathway (28). Gene inactivation experiments of *mvaA*, which encodes a class II 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, have shown that *mvaA* is essential for *in vitro* growth of *S. aureus* and that the mutant used is

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attenuated for virulence in a murine hematogenous pyelonephritis infection model (29).

In the various *S. aureus* genomes sequenced, seven identified genes are involved in the mevalonate pathway, based on sequence similarities and biochemical studies (30). The following reactions have been proposed: 1) condensation of two molecules of acetyl-CoA to form acetoacetyl-CoA, catalyzed by acetyl-CoA acetyltransferases; acetyl-CoA acetyltransferase homologues (SA0223) are present in various staphylococcal genomes; 2) Claisen condensation of acetyl-CoA with acetoacetyl-CoA to yield HMG-CoA, catalyzed by HMG-CoA synthase; the crystal structure and catalytic mechanism of the *S. aureus* HMG-CoA synthase (encoded by *mvaS*) have been investigated (30); 3) reduction of HMG-CoA to mevalonate, catalyzed by HMG-CoA reductase (encoded by *mvaA*) with NADPH as cofactor; HMG-CoA reductase is the best characterized enzyme of the mevalonate pathway in both eukaryotes and prokaryotes (29), and crystal structures have been solved for both human and bacterial HMG-CoA reductase; the eukaryotic HMG-CoA reductase is the target of the statin class of cholesterol-lowering agents; 4) Phosphorylation of mevalonate with ATP to form mevalonate-5-phosphate and then in a second reaction mevalonate diphosphate, catalyzed by mevalonate kinase; mevalonate kinase from *S. aureus* (*mvaK1*) has been characterized recently (31); 5) decarboxylation of mevalonate diphosphate to isopentenyl diphosphate, catalyzed by mevalonate-diphosphate decarboxylase (encoded by *mvaD*); 6) isomerization of isopentenyl diphosphate to produce dimethylallyl diphosphate in the presence of both FMN and NADPH, catalyzed by a type II isopentenyl-diphosphate isomerase (encoded by *fni*); this type II enzyme was first described by Kaneda *et al.* (32); and 7) condensation of isopentenyl diphosphate and geranyl diphosphate to form farnesyl diphosphate, catalyzed by farnesyl-PP synthase (encoded by *ispA*).

The mevalonate pathway used in humans is extended to synthesize lanosterol and finally cholesterol. In bacteria, end products include the lipid carrier undecaprenol, which is involved in cell wall biosynthesis, menaquinones and ubiquinones, which are involved in electron transport, and carotenoids, *e.g.* staphyloxanthin.

Staphyloxanthin is a typical secondary metabolite. It is not necessary for the growth and reproduction of *S. aureus* but might serve a role in survival in infected hosts and in combating the immune system.

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REFERENCES

1. Götz, F., Bannerman, T., and Schleifer, K. H. (2004) in *The Procaryotes* (Dworkin, M., ed) http://141.150.157.117:8080/prokWIP/chaphtm/356/COMPLETE.htm, Springer-Verlag, New York
2. Pinner, M., and Voldrich, M. (1932) *J. Infect. Dis.* **50,** 185–202
3. Marshall, J. H., and Wilmoth, G. J. (1981) *J. Bacteriol.* **147,** 900–913
4. Armstrong, G. A. (1997) *Annu. Rev. Microbiol.* **51,** 629–659
5. Wieland, B., Feil, C., Gloria-Maercker, E., Thumm, G., Lechner, M., Bravo, J. M., Poralla, K., and Götz, F. (1994) *J. Bacteriol.* **176,** 7719–7726
6. Götz, F., and Schumacher, B. (1987) *FEMS Microbiol. Lett.* **40,** 285–288
7. Kreutz, B., and Götz, F. (1984) *Gene* (*Amst.*) **31,** 301–304
8. Rosenstein, R., Peschel, A., Wieland, B., and Götz, F. (1992) *J. Bacteriol.* **174,** 3676–3683
9. Peschel, A., Augustin, J., Kupke, T., Stevanovic, S., and Götz, F. (1993) *Mol. Microbiol.* **9,** 31–39
10. Wieland, K. P., Wieland, B., and Götz, F. (1995) *Gene* (*Amst.*) **158,** 91–96
11. Kuroda, M., Ohta, T., Uchiyama, I., Baba, T., Yuzawa, H., Kobayashi, I., Cui, L., Oguchi, A., Aoki, K., Nagai, Y., Lian, J., Ito, T., Kanamori, M., Matsumaru, H., Maruyama, A., Murakami, H., Hosoyama, A., Mizutani-Ui, Y., Takahashi, N. K., Sawano, T., Inoue, R., Kaito, C., Sekimizu, K., Hirakawa, H., Kuhara, S., Goto, S., Yabuzaki, J., Kanehisa, M., Yamashita, A., Oshima, K., Furuya, K., Yoshino, C., Shiba, T., Hattori, M., Ogasawara, N., Hayashi, H., and Hiramatsu, K. (2001) *Lancet* **357,** 1225–1240
12. Kullik, I., Giachino, P., and Fuchs, T. (1998) *J. Bacteriol.* **180,** 4814–4820
13. Lang, S., Livesley, M. A., Lambert, P. A., Littler, W. A., and Elliott, T. S. (2000) *FEMS Immunol. Med. Microbiol.* **29,** 213–220
14. Takami, H., Takaki, Y., and Uchiyama, I. (2002) *Nucleic Acids Res.* **30,** 3927–3935
15. Takaichi, S., Inoue, K., Akaike, M., Kobayashi, M., Oh-oka, H., and Madigan, M. T. (1997) *Arch. Microbiol.* **168,** 277–281
16. Botella, J. A., Murillo, F. J., and Ruiz-Vazquez, R. (1995) *Eur. J. Biochem.* **233,** 238–248
17. Armstrong, G. A., Alberti, M., and Hearst, J. E. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 9975–9979
18. Schumann, G., Nurnberger, H., Sandmann, G., and Krugel, H. (1996) *Mol. Gen. Genet.* **252,** 658–666
19. Rocap, G., Larimer, F. W., Lamerdin, J., Malfatti, S., Chain, P., Ahlgren, N. A., Arellano, A., Coleman, M., Hauser, L., Hess, W. R., Johnson, Z. I., Land, M., Lindell, D., Post, A. F., Regala, W., Shah, M., Shaw, S. L., Steglich, C., Sullivan, M. B., Ting, C. S., Tolonen, A., Webb, E. A., Zinser, E. R., and Chisholm, S. W. (2003) *Nature* **424,** 1042–1047
20. Eisen, J. A., Nelson, K. E., Paulsen, I. T., Heidelberg, J. F., Wu, M., Dodson, R. J., Deboy, R., Gwinn, M. L., Nelson, W. C., Haft, D. H., Hickey, E. K., Peterson, J. D., Durkin, A. S., Kolonay, J. L., Yang, F., Holt, I., Umayam, L. A., Mason, T., Brenner, M., Shea, T. P., Parksey, D., Nierman, W. C., Feldblyum, T. V., Hansen, C. L., Craven, M. B., Radune, D., Vamathevan, J., Khouri, H., White, O., Gruber, T. M., Ketchum, K. A., Venter, J. C., Tettelin, H., Bryant, D. A., and Fraser, C. M. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99,** 9509–9514
21. Cramton, S. E., Gerke, C., Schnell, N. F., Nichols, W. W., and Götz, F. (1999) *Infect. Immun.* **67,** 5427–5433
22. Gerke, C., Kraft, A., Süssmuth, R., Schweitzer, O., and Götz, F. (1998) *J. Biol. Chem.* **273,** 18586–18593
23. Heilmann, C., Schweitzer, O., Gerke, C., Vanittanakom, N., Mack, D., and Götz, F. (1996) *Mol. Microbiol.* **20,** 1083–1091
24. Saxena, I. M., Brown, R. M., Jr., Fevre, M., Geremia, R. A., and Henrissat, B. (1995) *J. Bacteriol.* **177,** 1419–1424
25. Peschel, A., Ottenwälder, B., and Götz, F. (1996) *FEMS Microbiol. Lett.* **137,** 279–284
26. Marshall, J. H., and Wilmoth, G. J. (1981) *J. Bacteriol.* **147,** 914–919
27. Moss, G. P. (1979) *Pure Appl. Chem.* **51,** 507–514
28. Wilding, E. I., Brown, J. R., Bryant, A. P., Chalker, A. F., Holmes, D. J., Ingraham, K. A., Iordanescu, S., So, C. Y., Rosenberg, M., and Gwynn, M. N. (2000) *J. Bacteriol.* **182,** 4319–4327
29. Wilding, E. I., Kim, D. Y., Bryant, A. P., Gwynn, M. N., Lunsford, R. D., McDevitt, D., Myers, J. E., Jr., Rosenberg, M., Sylvester, D., Stauffacher, C. V., and Rodwell, V. W. (2000) *J. Bacteriol.* **182,** 5147–5152
30. Campobasso, N., Patel, M., Wilding, I. E., Kallender, H., Rosenberg, M., and Gwynn, M. N. (2004) *J. Biol. Chem.* **279,** 44883–44888
31. Voynova, N. E., Rios, S. E., and Miziorko, H. M. (2004) *J. Bacteriol.* **186,** 61–67
32. Kaneda, K., Kuzuyama, T., Takagi, M., Hayakawa, Y., and Seto, H. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 932–937
33. Marshall, J. H., and Rodwell, E. S. (1972) *3rd International Symposium on Carotenoids Other Than Vitamin A, September 4–7,* 1972, pp. 56–57, International Union of Pure and Applied Chemisry, Cluj, Romania

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Search Method for Inhibitors of Staphyloxanthin Production by Methicillin-Resistant *Staphylococcus aureus*

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Staphyloxanthin, a yellow pigment produced by methicillin-resistant *Staphylococcus aureus* (MRSA), is a virulent factor escaping from the host immune system. A new screening method for inhibitors of staphyloxanthin production by MRSA was established using paper disks. By this screening method, inhibitors of staphyloxanthin production were selected from the natural product library (*ca.* 300) and from actinomycete culture broths (*ca.* 1000). From the natural product library, four known inhibitors of lipid metabolism, cerulenin, dihydrobisvertinol, xanthohumol and zaragozic acid, were found to inhibit staphyloxanthin production; however, typical antibiotics used clinically, including vancomycin, had no effect on staphyloxanthin production. From actinomycete culture broths, two known anthraquinones, 6-deoxy-8-*O*-methylrabelomycin and tetrangomycin, were found to inhibit staphyloxanthin production by MRSA in the paper disk assay. These results suggested that this screening method is useful and effective to find compounds targeting staphyloxanthin production, leading to a new type of chemotherapeutics against MRSA infection.

Key words methicillin-resistant *Staphylococcus aureus*: staphyloxanthin: screening method: microbial metabolite

Methicillin-resistant *Staphylococcus aureus* (MRSA) is recognized as a major nosocomial pathogen which is resistant to all current β-lactams.[1] Vancomycin is widely used to treat MRSA infection; however, vancomycin-resistant *S. aureus* has been identified.[2] It is therefore important and necessary to discover and develop new anti-MRSA agents.

It is well known that MRSA produces a yellow pigment called staphyloxanthin.[3,4] Recently, several research groups reported that staphyloxanthin is a virulent factor acting as an antioxidant, with its numerous conjugated double bonds enabling detoxification of host immune system-generated reactive oxygen species.[5—7] The first committed step in staphyloxanthin biosynthesis by *S. aureus* is dehydrosqualene synthase (CrtM) enzyme, which catalyzes the condensation of two farnesyl diphosphates to produce the C30 species, presqualene diphosphate.[7] A CrtM-deficient mutant cannot produce staphyloxanthin to fail in survival in the host mouse.[5] It suggests that if we can control or inhibit the production of staphyloxanthin by small molecules, MRSA will be unable to infect and survive in the host. Thus, the staphyloxanthin biosynthetic pathway of MRSA is a potential target to combat MRSA infection. Recently, farnesol[8] was reported to inhibit staphyloxanthin production by MRSA.[9]

Based on this new concept, we established convenient assay systems to observe staphyloxanthin production by MRSA and started to search for inhibitors of staphyloxanthin production from natural sources. During this screening program, cerulenin (an inhibitor of fatty acid synthase),[10] dihydrobisvertinol,[11,12] xanthohumol (an inhibitor of diacylglycerol acyltransferase)[13,14] and zaragozic acid (an inhibitor of squalene synthase)[15] (Fig. 1), which are known as inhibitors of lipid metabolism, were selected from our natural product library. Moreover, five anthraquinones, SM-196B (**1**),[16] 1-deoxo-1-hydroxy-8-*O*-methylrabelomycin (**2**),[17] 8-*O*-methylrabelomycin (**3**),[18] 6-deoxy-8-*O*-methylrabelomycin (**4**)[18] and tetrangomycin (**5**)[19] (Fig. 1), previously reported as antibiotics,[16—19] were isolated from the culture broth of actinomycete strain *Streptomyces badius* 4—6. Among them, **4** and **5** showed inhibitory activity of MRSA staphyloxanthin production in the paper disk assay. In this study, we describe the assay systems to evaluate staphyloxanthin production by MRSA and the inhibitory activity of microbial metabolites against pigment production.

MATERIALS AND METHODS

Materials Vancomycin, tetracycline, ciprofloxacin and farnesol were purchased from Wako Junyaku Co., Ltd. (Osaka, Japan). Streptomycin and arbekacin were purchased from Meiji Seika Pharma Co., Ltd. (Tokyo, Japan). Imipenem was purchased from Banyu Pharmaceutical Co., Ltd. (Tokyo, Japan). Erythromycin was isolated from microbial sources by our group.

Media Mueller–Hinton Broth (MHB), Todd Hewitt Broth (THB), yeast extract, bacto tryptone and bacto agar were purchased from Becton, Dickinson and Co. (Franklin Lakes, NJ, U.S.A.). Glucose and K_2HPO_4 were purchased from Wako Junyaku Co., Ltd. (Osaka, Japan). Glycerol, soluble starch and NaCl were purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). Glycerol monoacetate was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Pharmamedia was purchased from IWAKI and Co., Ltd. (Tokyo, Japan). Diaion HP-20 was purchased from Mitsubishi Chemical Co., Ltd. (Tokyo, Japan). Taiyo-agar was purchased from Shimizu Shokuhin Kaisha Ltd. (Shizuoka, Japan).

A-3M medium included 0.5% glucose, 2.0% glycerol, 2.0% soluble starch, 1.5% pharmamedia, 0.3% yeast extract and 1.0% Diaion HP-20 (pH 7.0). Tryptone yeast extract broth (TYB) medium[3] included 1.7% bacto tryptone, 1.0% yeast extract, 0.5% NaCl, 0.25% K_2HPO_4 and 1.5% glycerol monoacetate. LA medium included 1.0% bacto tryptone, 0.5% yeast extract, 1.0% NaCl and 1.5% Taiyo-agar. THB agar consisted

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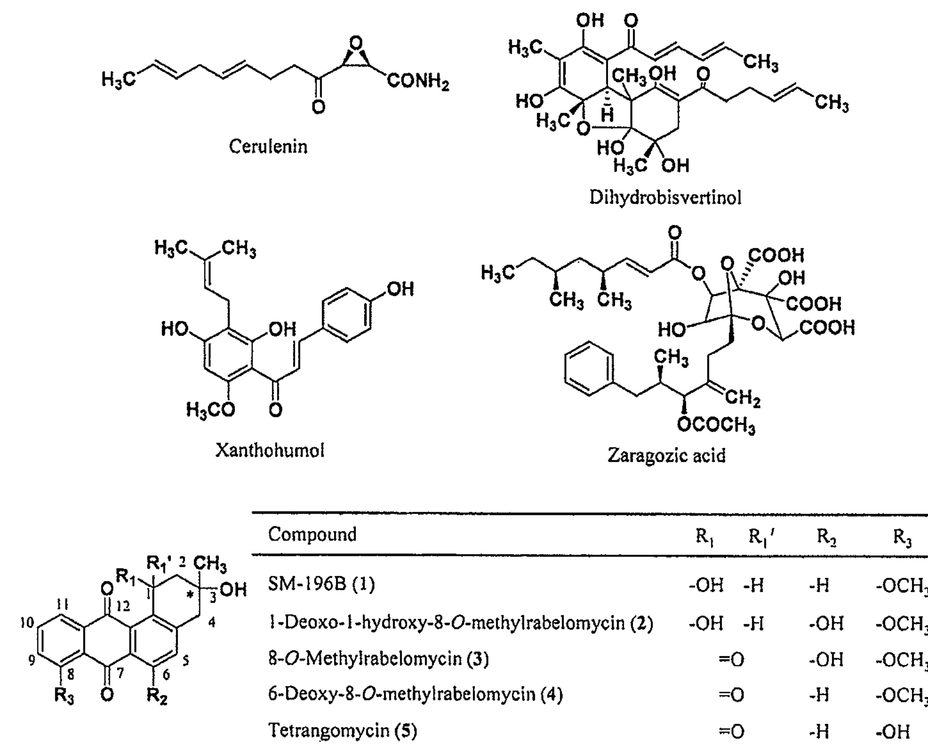

Compound	R_1	R_1'	R_2	R_3
SM-196B (1)	-OH	-H	-H	$-OCH_3$
1-Deoxo-1-hydroxy-8-*O*-methylrabelomycin (2)	-OH	-H	-OH	$-OCH_3$
8-*O*-Methylrabelomycin (3)	=O		-OH	$-OCH_3$
6-Deoxy-8-*O*-methylrabelomycin (4)	=O		-H	$-OCH_3$
Tetrangomycin (5)	=O		-H	-OH

Fig. 1. Structures of Lipid Metabolism Inhibitors and Five Anthraquinones 1 to 5 Selected as Inhibitors of Yellow Pigment Production by MRSA in the Paper Disk Assay

of THB plus with 1.5% bacto agar, and TYB agar consisted of TYB plus with 1.5% bacto agar.

Microorganisms MRSA K-24 strain, a clinical isolate, was used as a yellow pigment-producing strain. Actinomycetes including *S. badius* 4—6 strain were isolated from soil collected on Hegurajima Island, Ishikawa, Japan. *S. badius* 4—6 strain was used for the production of five anthraquinones **1** to **5**.

General Methods for Structural Determination ^{1}H-NMR spectra were recorded on a Varian Mercury XL-400 NMR spectrometer (Varian, Inc., Palo Alto, CA, U.S.A.). Chemical shifts are shown as δ values (ppm) relative to $CDCl_3$ at δ 7.26 ppm for ^{1}H-NMR. FAB-mass spectra were read on a JMS-DX300 spectrometer (JEOL, Tokyo, Japan).

Microbial Samples Our natural product library consists of more than 300 compounds, including cerulenin, dihydrobisvertinol, xanthohumol, zaragozic acid and unpublished compounds, mainly of microbial origin.

The actinomycetes were shaken in A-3M medium at 30°C for 6 d. After the culture broths had been treated with butanol, the butanol extracts were dried *in vacuo*. The extracts were dissolved in 50% ethanol and used as screening samples. Anthraquinones **1** (33.9 mg), **2** (8.8 mg), **3** (2.7 mg), **4** (8.1 mg) and **5** (0.7 mg), were isolated as yellow powders from the culture broth (1.0 L) of *S. badius*.

SM-196B (**1**): FAB-MS: 338 $[M]^+$; ^{1}H-NMR in $CDCl_3$ δ: 1.49 (3H, s), 2.30 (2H, ddd), 3.00 (2H, dd), 4.02 (3H, s), 5.44 (1H, t), 7.28 (1H, d), 7.48 (1H, d), 7.68 (1H, t), 7.84 (1H, d), 8.18 (1H, d). These data are identical to those reported previously.[16]

1-Deoxo-1-hydroxy-8-*O*-methylrabelomycin (**2**): FAB-MS: 354 $[M]^+$; ^{1}H-NMR in $CDCl_3$ δ: 1.50 (3H, s), 2.22 (2H, ddd), 2.98 (2H, dd), 4.04 (3H, s), 5.38 (1H, t), 7.04 (1H, s), 7.36 (1H, d), 7.75 (1H, t), 7.90 (1H, d), 13.28 (1H, s). These data are identical to those reported previously.[17]

8-*O*-Methylrabelomycin (**3**): FAB-MS: 352 $[M]^+$; ^{1}H-NMR in $CDCl_3$ δ: 1.48 (3H, s), 2.96 (2H, dd), 3.05 (2H, s), 4.04 (3H, s), 6.98 (1H, s), 7.32 (1H, dd), 7.72 (1H, dd), 7.73 (1H, t), 13.03 (1H, s). These data are identical to those reported previously.[18]

6-Deoxy-8-*O*-methylrabelomycin (**4**): FAB-MS: 336 $[M]^+$; ^{1}H-NMR in $CDCl_3$ δ: 1.44 (3H, s), 2.98 (2H, dd), 3.20 (2H, dd), 4.02 (3H, s), 7.51 (1H, dd), 7.65 (1H, dd), 7.66 (1H, dd), 7.80 (1H, t), 8.25 (1H, d). These data are identical to those reported previously.[18]

Tetrangomycin (**5**): FAB-MS: 322 $[M]^+$; ^{1}H-NMR in $CDCl_3$ δ: 1.45 (3H, s), 3.05 (2H, dd), 3.49 (2H, dd), 7.21 (1H, dd), 7.49 (1H, dd), 7.64 (1H, dd), 7.66 (1H, t), 8.30 (1H, d). These data are identical to those reported previously.[19]

Assay Methods for Staphyloxanthin Production by MRSA Paper disk method; MRSA was cultured in MHB at 37°C for 20 h and adjusted to 1×10^8 colony forming unit (CFU)/mL. The inoculum (100 μL) was spread on TYB agar (25 mL) on a plate (STERILE No. 2 SQUARE SCHALE, 100×140 mm; Eiken Chemical Co., Ltd., Tochigi, Japan). After incubation at 37°C for 4 h, paper disks (6 mm i.d.; Toyo Roshi Kaisha Ltd., Tokyo, Japan) containing various amounts of a sample were placed on the plate and incubated at 37°C for 68 h. Inhibition of the production of yellow pigments, mainly

staphyloxanthin, in MRSA by a sample is expressed as the diameter (mm) of the white zone on the plate. The white zone indicates that the production of yellow pigments is selectively inhibited without any effect on MRSA growth. In some cases, the inhibitory zone of a sample against MRSA was observed inside the white zone at the same time. This method was used for screening.

Liquid culture method; A mixture containing TYB (980μL), a sample (10μL) and MRSA (10μL) at a final concentration of 1×10^7CFU/mL in a total volume of 1000μL was incubated at 210 rpm for 72 h at 37°C. 1) Effect on MRSA growth: The culture's turbidity was determined at 600 nm using a Power Wave×340 (BIO-TEK Instruments Inc., Vermont, U.S.A.). 2) Effect on yellow pigment production: After the culture was centrifuged, yellow pigments of the cell fraction were extracted with methanol (500μL) at 60°C for 2 h in the dark. The absorbance of yellow pigments was determined at 450 nm using a Power Wave×340. Inhibition of MRSA growth and yellow pigment production by a sample (% of control) is defined as (absorbance-sample/absorbance-control)×100. The IC_{50} value is defined as a sample concentration that causes 50% inhibition of MRSA growth and yellow pigment production, respectively.

Detection of Staphyloxanthin by TLC Detection of staphyloxanthin by TLC was described previously.[9] Briefly, MRSA grown under liquid cultures was collected and yellow pigments were extracted with methanol. Methanol extracts were evaporated in the dark. The dried samples were dissolved in methanol (50μL) and the extracts (10μL) were spotted and separated on a TLC (HPTLC silica gel 60, 0.5 mm thick, 10×10 cm; Merck, Hessen, Germany) with chloroform/methanol/water (65:25:4, v/v/v) as a developing solvent. The pigment spots were observed visually. *Rf* value of staphyloxanthin was 0.8 under this condition.

RESULTS

Establishment of Screening Method Medium for Staphyloxanthin Production by MRSA: Three agar media were compared for yellow pigment production by MRSA. MRSA was spread on LA medium, THB agar or TYB agar in a plate. After incubation at 37°C for 72 h, MRSA produced yellow pigments on TYB agar, but not on LA medium or THB agar (Table 1A). Thus, the TYB agar was selected for subsequent experiments.

Concentration of Glycerol Monoacetate: Glycerol monoacetate is an essential factor for yellow pigment production by MRSA.[3] MRSA cultured in MHB was incubated on TYB agar supplemented with 0—2.0% glycerol monoacetate on a plate. As a result, yellow pigments were observed clearly when MRSA was incubated at 37°C for 72 h in the presence of 1.5% glycerol monoacetate (Table 1B). Thus, the concentration of 1.5% glycerol monoacetate was adopted for this assay.

Placement Time of Samples: MRSA cultured in MHB was spread on TYB agar. After incubation at 37°C for 0—10 h, paper disks containing vancomycin (5μg), arbekacin (5μg) or farnesol (10μg) were placed on TYB agar and incubated at 37°C until 72 h after MRSA inoculation. Vancomycin and arbekacin, as known clinical anti-MRSA agents, inhibited the growth of MRSA when they were placed at time 0 (at the same time as MRSA inoculation); however, when they were placed 4—10 h after MRSA inoculation, they showed almost no effect on the growth of MRSA (Fig. 2). On the other hand, farnesol inhibited yellow pigment production at 0—7 h with almost no effect on MRSA growth at 4—7 h (Fig. 2). Microbial culture broths usually contain various compounds with a different mechanism of action. If a microorganism produces an anti-MRSA compound and an inhibitor of yellow pigment production, such a culture broth may show only inhibitory zone when the paper disk is placed on the MRSA plate at time 0. To reduce the effect of anti-MRSA compounds on the production of yellow pigments in the first screening assay, the placement time of paper disks containing samples was set up at 4 h after MRSA incubation. Under this condition, in fact, vancomycin and arbekacin showed almost no inhibitory zone on the MRSA plate.

Effect of Typical Antibiotics on This Assay: Effects of 7 representative antibiotics on the growth of MRSA and on yellow pigment production by MRSA were investigated (Table 2). Arbekacin, streptomycin and vancomycin (each 5μg/disk) showed an inhibitory zone (growth inhibition of MRSA), but none of the antibiotics tested showed a white zone (inhibition of yellow pigment production) around paper disks.

Table 1. (A) Effect of Three Media on Yellow Pigment Production

Medium	Color
LA medium	Colorless
THB agar	Colorless
TYB agar	Pale yellow

Table 1. (B) Effect of Glycerol Monoacetate's Concentration in TYB Agar on Yellow Pigment Production

Concentration	Color
0.0%	Colorless
0.5%	Colorless
1.0%	Pale yellow
1.5%	Yellow
2.0%	Pale yellow

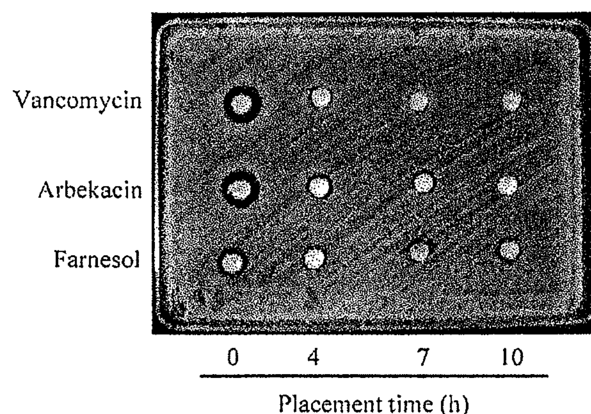

Fig. 2. Determination of Sample Placement Time

Paper disks (6 mm i.d.) containing vancomycin (5μg), arbekacin (5μg) or farnesol (10μg) were placed on TYB agar at 0, 4, 7 and 10 h after MRSA inoculation, and incubated at 37°C. The diameters of inhibitory or white zones were measured at 72 h after MRSA inoculation.

Screening Results From the natural product library (about 300 compounds), four compounds, cerulenin,[10] dihydrobisvertinol,[11] xanthohumol[13] and zaragozic acid,[15] were selected (Table 3). All of these compounds have been reported as inhibitors of lipid metabolism;[10—15] however, among inhibitors of lipid metabolism, lovastatin and triclosan had no effect on yellow pigment production.

From actinomycete culture broths (about 1000 culture broths), a culture broth of *S. badius* was selected. Five structurally related compounds were isolated from the culture broth and identified as known anthraquinones, 1 to 5.[16—19] Among them, 4 and 5 were found to show dose-dependent white zones (9, 11 mm for 4 and 18, 26 mm for 5) at 5.0 and 10 μg/disk, respectively (Table 3). Thus, 5 exhibited potent inhibition of yellow pigment production with this assay, but also showed growth inhibition at 10 μg/disk. Anthraquinone 3 showed only growth inhibition at 20 μg/disk, and 1 and 2 showed no effect on yellow pigment production and MRSA growth even at 20 μg/disk (Table 3).

Table 2. Effect of Antibiotics on MRSA Growth and Yellow Pigment Production

Antibiotics[a]	Inhibitory zone (mm)	White zone (mm)
Arbekacin	8	—
Ciprofloxacin	—	—
Erythromycin	—	—
Imipenem	—	—
Streptomycin	8	—
Tetracycline	—	—
Vancomycin	7	—

a) Concentration: 5 μg/disk.

Effect of Two Anthraxquinones on the Production of Staphyloxanthin by MRSA Since 4 and 5 showed white zones in the paper disk assay, they were evaluated in the liquid culture assay to see the selectivity toward the yellow pigment production. Therefore, the IC_{50} values of 4 and 5 against the growth and yellow pigment production of MRSA were measured in the liquid culture. Accordingly, selectivity index (SI, IC_{50} value against MRSA growth/IC_{50} value against yellow pigment production) was also calculated. As shown in Fig. 3 and Table 4, the IC_{50} values of 4 and 5 against the growth were 48 and 3.5 μg/mL, and the IC_{50} values against yellow pigment production were 6.0 and 4.4 μg/mL, respectively. As a result, SI values were calculated to be 8.0 and 0.8, indicating that 4 showed high selectivity toward pigment production in the liquid culture assay (Table 4).

Thus, 4 was found to show a white zone in the paper disk assay and to exhibit selectivity in inhibition of yellow pigment production in the liquid culture assay. To investigate whether staphyloxanthin production by MRSA was inhibited by the anthraquinone, staphyloxanthin was analyzed by TLC.[9] As shown in Fig. 4, the spot corresponding to staphyloxanthin (*Rf* 0.8) completely disappeared at 50 μg/mL of the compound 4.

DISCUSSION

As described in this study, we established assay methods to evaluate yellow pigment production by MRSA, and screened for inhibitors of yellow pigment production from natural sources by the paper disk method. From the natural product library, four inhibitors of lipid metabolism (cerulenin, dihydrobisvertinol, xanthohumol and zaragozic acid) and from actinomycete culture broths two anthraquinones 4 and 5 were isolated. Staphyloxanthin consists of a polyprenyl moiety, a

Table 3. Effect of Candidate Inhibitors on MRSA Growth and Yellow Pigment Production

Compound	Concentration (μg/disk)	Inhibitory zone (mm)	White zone (mm)
Cerulenin	2.5	—	9
	5	—	12
	10	9	15
Dihydrobisvertinol	5	—	8
Xanthohumol	5	—	9
Zaragozic acid	10	—	—
	50	—	10
	100	—	16
SM-196B (1)	20	—	—
1-Deoxo-1-hydroxy-8-*O*-methylrabelomycin (2)	20	—	—
8-*O*-Methylrabelomycin (3)	20	8	—
6-Deoxy-8-*O*-methylrabelomycin (4)	5	—	9
	10	—	11
Tetrangomycin (5)	5	—	18
	10	11	26

Table 4. IC_{50} and SI Values of Two Anthraquinones in Liquid Method

Compound	IC_{50} (μg/mL)[a]		SI[b]
	MRSA growth	Yellow pigment production	
6-Deoxy-8-*O*-methylrabelomycin (4)	48	6	8
Tetrangomycin (5)	3.5	4.4	0.8

a) Liquid culture method. *b*) Selectivity index (SI): IC_{50} against MRSA growth/IC_{50} against yellow pigment production.

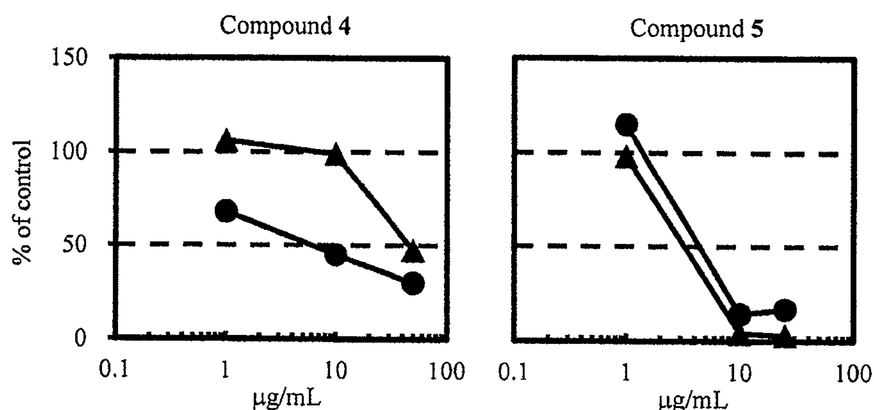

Fig. 3. Effect of Two Compounds on MRSA Growth and Yellow Pigment Production in the Liquid Culture Assay

MRSA was cultured in the presence of 4 and 5 (0—50 µg/mL) for 72 h. The growth of MRSA (▲) was calculated by measuring the absorbance at 600 nm. These yellow pigments were extracted with methanol as described in Materials and Methods. The production of yellow pigment (●) was calculated by measuring the absorbance at 450 nm.

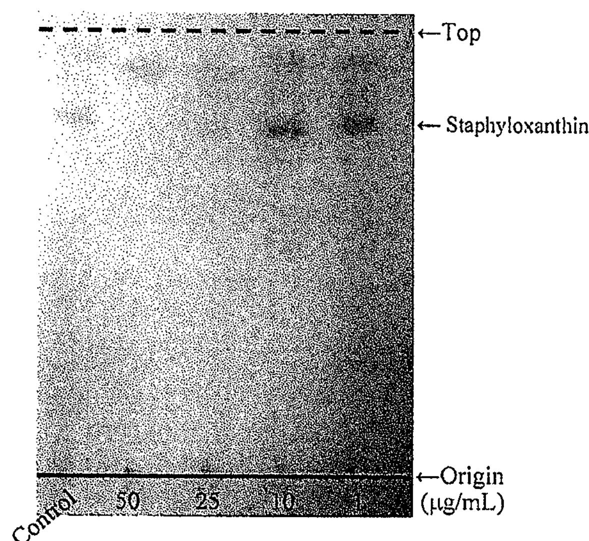

Fig. 4. Inhibition of Staphyloxanthin Production in MRSA by 4

MRSA was cultured in the presence of 4 (0, 1, 10, 25, 50 µg/mL) for 72 h. These yellow pigments were extracted with methanol and separated on TLC according to the established method.[9]

sugar moiety and an acyl moiety; therefore, it seems reasonable that these inhibitors of lipid metabolism were selected as showing a white zone in the paper disk method, since they inhibited the steps of polyprenyl and acyl residue production in the staphyloxanthin structure. Two anthraquinones, originally reported as antibiotics,[18,19] were found to inhibit production of yellow pigment in MRSA. In particular, 4 has high selectivity in the inhibition of yellow pigment production (Table 4). Furthermore, we confirmed that staphyloxanthin production was inhibited by the compound (Fig. 4). The mechanism of action of 4 remains to be studied.

From the biological data (Tables 3 and 4) of structurally related anthraquinones, it was suggested that the presence of a ketone at C-1 (R_1) and a proton at C-6 (R_2) are essential for inhibition of yellow pigment production by MRSA.

Anthraquinone 5 showed potent inhibition of yellow pigment production in the paper disk assay, but low selectivity in the liquid culture method. The reason might be as follows. Compound 5 has an intrinsic anti-MRSA activity by inhibiting early stage of the growth. In the paper disk assay, the disk containing 5 was placed on MRSA at 4 h after inoculation, while 5 was added from the beginning of the MRSA culture in the liquid culture assay.

Our screening method using paper disks proved useful in the search for new inhibitors of staphyloxanthin biosynthesis. It is expected that this screening method would have the following advantages: 1) It is a convenient assay system to observe inhibition of MRSA staphyloxanthin production as a white zone; 2) Inhibitors of steps (enzymes) involved in staphyloxanthin biosynthesis, including CrtM, CrtN and so on[5] can be found; 3) Inhibitors of lipid metabolism and potential polyprenyl transferase or acyl transferase to the sugar moiety might be found. Thus, this strategy could lead to the discovery of new compounds against MRSA infection.

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REFERENCES

1) Centers for Disease Control and Prevention (CDC). *Staphylococcus aureus* with reduced susceptibility to vancomycin—United States, 1997. *MMWR Morb. Mortal. Wkly. Rep.*, 46, 765—766 (1997).
2) Hiramatsu K, Hanaki H, Ino T, Yabuta K, Oguri T, Tenover FC. Methicillin-resistant *Staphylococcus aureus* clinical strain with reduced vancomycin susceptibility. *J. Antimicrob. Chemother.*, 40, 135—136 (1997).
3) Marshall JH, Wilmoth GJ. Pigments of *Staphylococcus aureus*, a series of triterpenoid carotenoids. *J. Bacteriol.*, 147, 900—913 (1981).
4) Marshall JH, Wilmoth GJ. Proposed pathway of triterpenoid carotenoid biosynthesis in *Staphylococcus aureus*: evidence from a study of mutants. *J. Bacteriol.*, 147, 914—919 (1981).
5) Liu GY, Essex A, Buchanan JT, Datta V, Hoffman HM, Bastian JF, Fierer J, Nizet V. *Staphylococcus aureus* golden pigment impairs

neutrophil killing and promotes virulence through its antioxidant activity. *J. Exp. Med.*, **202**, 209—215 (2005).

6) Clauditz A, Resch A, Wieland KP, Peschel A, Götz F. Staphyloxanthin plays a role in the fitness of *Staphylococcus aureus* and its ability to cope with oxidative stress. *Infect. Immun.*, **74**, 4950—4953 (2006).

7) Liu CI, Liu GY, Song Y, Yin F, Hensler ME, Jeng WY, Nizet V, Wang AH, Oldfield E. A cholesterol biosynthesis inhibitor blocks *Staphylococcus aureus* virulence. *Science*, **319**, 1391—1394 (2008).

8) Yamamoto H, Yokoyama H, Simpson K, Nakayama TOM, Chichester CO. Incorporation of 5,10,15 ^{14}C-farnesol pyrophosphate into *Phycomyces* carotenoids. *Nature*, **191**, 1299—1300 (1961).

9) Kuroda M, Nagasaki S, Ohta T. Sesquiterpene farnesol inhibits recycling of the C55 lipid carrier of the murein monomer precursor contributing to increased susceptibility to beta-lactams in methicillin-resistant *Staphylococcus aureus*. *J. Antimicrob. Chemother.*, **59**, 425—432 (2007).

10) Ōmura S. The antibiotic cerulenin, a novel tool for biochemistry as an inhibitor of fatty acid synthesis. *Bacteriol. Rev.*, **40**, 681—697 (1976).

11) Trifonov LS, Hilpert H, Floersheim P, Dreiding AS, Rast DM, Skrivanova R, Hoesch L. Bisvertinols: a new group of dimeric vertinoids from *Verticillium intertextum*. *Tetrahedron*, **42**, 3157—3179 (1986).

12) Koyama N, Ohshiro T, Tomoda H, Ōmura S. Fungal isobisvertinol, a new inhibitor of lipid droplet accumulation in mouse macrophages. *Org. Lett.*, **9**, 425—428 (2007).

13) Tobe H, Muraki Y, Kitamura K, Komiyama O, Sato Y, Sugioka T, Maruyama HB, Matsuda E, Nagai M. Bone resorption inhibitors from hop extract. *Biosci. Biotechnol. Biochem.*, **61**, 158—159 (1997).

14) Tabata N, Ito M, Tomoda H, Ōmura S. Xanthohumols, diacylglycerol acyltransferase inhibitors, from *Humulus lupulus*. *Phytochemistry*, **46**, 683—687 (1997).

15) Bergstrom JD, Kurtz MM, Rew DJ, Amend AM, Karkas JD, Bostedor RG, Bansal VS, Dufresne C, VanMiddlesworth FL, Hensens OD, Liesh JM, Zink DL, Wilson KE, Onishi J, Milligan JA, Bills G, Kaplan L, Nallin Omstead M, Jenkins RG, Huang L, Meinz MS, Quinn L, Burg RW, Kong YL, Mochales S, Mojena M, Martin I, Pelaez F, Diez MT, Alberts AW. Zaragozic acids: a family of fungal metabolites that are picomolar competitive inhibitors of squalene synthase. *Proc. Natl. Acad. Sci. U.S.A.*, 90, 80—84 (1993).

16) Grabley S, Hammann P, Hütter K, Kluge H, Thiericke R, Wink J, Zeeck A. Secondary metabolites by chemical screening. Part 19. SM 196 A and B, novel biologically active angucyclinones from *Streptomyces* sp. *J. Antibiot.*, **44**, 670—673 (1991).

17) Fotso S, Mahmud T, Zabriskie TM, Santosa DA, Proteau PJ. Rearranged and unrearranged angucyclinones from Indonesian *Streptomyces* spp. *J. Antibiot.*, **61**, 449—456 (2008).

18) Shigihara Y, Koizumi Y, Tamamura T, Homma Y, Isshiki K, Dobashi K, Naganawa H, Takeuchi T. 6-Deoxy-8-*O*-methylrabelomycin and 8-*O*-methylrabelomycin from a *Streptomyces* species. *J. Antibiot.*, **41**, 1260—1264 (1988).

19) Dann M, Lefemine DV, Barbatschi F, Shu P, Kunstmann MP, Mitscher LA, Bohonos N. Tetrangomycin, a new quinone antibiotic. *Antimicrob. Agents Chemother.* (Bethesda), **5**, 832—835 (1965).

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EXHIBIT 10

Vol. 74, No. 8

Staphyloxanthin Plays a Role in the Fitness of *Staphylococcus aureus* and Its Ability To Cope with Oxidative Stress

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Staphyloxanthin is a membrane-bound carotenoid of *Staphylococcus aureus*. Here we studied the interaction of staphyloxanthin with reactive oxygen substances (ROS) and showed by comparative analysis of the wild type (WT) and an isogenic *crtM* mutant that the WT is more resistant to hydrogen peroxide, superoxide radical, hydroxyl radical, hypochloride, and neutrophil killing.

Staphyloxanthin is an orange-red triterpenoid carotenoid whose biosynthesis and structure have been recently elucidated (14, 18). It is well known that carotenoids function as antioxidants, and it has been suggested that staphyloxanthin can protect *Staphylococcus aureus* against oxidative stress (12, 13). To further study the protective function of staphyloxanthin, we compared the viability of staphyloxanthin-producing *S. aureus* Newman (3) with that of its isogenic *crtM* mutant (19) (Fig. 1), which does not produce staphyloxanthin (but can be complemented by the expression plasmid pTX*crtM*) (14), to various radical and nonradical substances generated in vitro. We also tested the ability of staphyloxanthin to act as a radical scavenger.

First we investigated the oxidation of staphyloxanthin by hydroxyl radicals ($OH^{\cdot}$) and peroxynitrite ($ONOO^{-}$). We generated hydroxyl radicals by the Fenton reaction in an assay containing iron(II) chloride and hydrogen peroxide in equimolar concentrations ranging from 0.01 to 0.5 mM. Although the system is nonspecific, $OH^{\cdot}$ is likely to be among the oxidants produced, including $OOH^{\cdot}$ and $CH3^{\cdot}$ (20). Purified staphyloxanthin (14) in the reaction mixture was rapidly oxidized, as indicated by the decrease in absorbance at 478 nm (Fig. 2A). The absorbance of controls lacking iron(II) chloride, H_2O_2, or both did not decrease. The time course of staphyloxanthin oxidation in the test samples shows that after 2 min of incubation with 0.05, 0.1, and 0.5 mM equimolar amounts of iron chloride and hydrogen peroxide, 40, 50, and 60% of the staphyloxanthin was oxidized, respectively (Fig. 2C). The oxidation of staphyloxanthin by peroxynitrite was monitored for 3 h by monitoring the decrease in absorbance at the absorption maximum of staphyloxanthin in ethanol (470 nm). In this experiment, prolonged incubations were necessary because the concentration of peroxynitrite increased only gradually after addition of SIN-1. Staphyloxanthin oxidation by peroxynitrite anions was indicated by the decrease in absorption and at the same time the shift of the absorption maximum to shorter wavelengths, which indicates the reduction of double bonds (Fig. 2B). The absorbance of the controls lacking SIN-1 did not decrease.

Next we tested the viability of *S. aureus* after incubation with H_2O_2. The numbers of viable *S. aureus* Newman wild-type (WT) and *crtM* mutant cells decreased with increasing concentrations of H_2O_2 (50, 75, 100, and 150 mM) but to different extents (Fig. 3A). The number of CFU of the controls lacking H_2O_2 did not change. The survival of the bacteria in the test samples correlated with the H_2O_2 concentration. Both strains were susceptible to high concentrations of H_2O_2, but the *crtM* mutant was more susceptible than the WT at all concentrations. The viability of *S. aureus* was also investigated after incubation with superoxide radicals generated by both hypoxanthine and xanthine oxidase (XO) and phenazine methosulfate (PMS) and NADH. The generation of the radicals was monitored by measuring the reduction of cytochrome *c*. WT and the *crtM* mutant *S. aureus* cells were both sensitive to killing by hypoxanthine-XO (Fig. 3B), but the WT always revealed a better survival frequency independent of the presence of catalase, which increases XO activity, which is inhibited by H_2O_2 (Fig. 3B). The number of CFU of the control did not

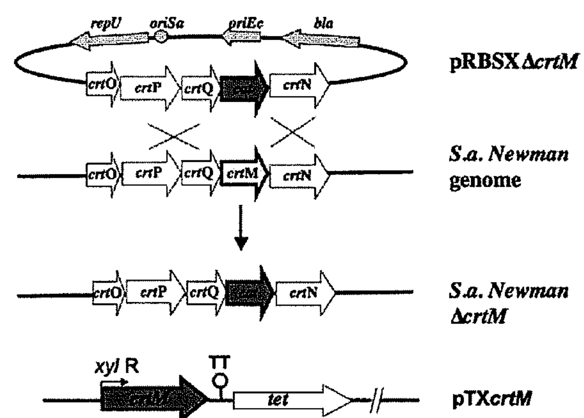

FIG. 1. Illustration of the construction of knockout plasmid pRBSXΔ*crtM* and xylose-inducible *crtM* expression plasmid pTX*crtM*, which is able to complement the *crtM* mutant in the presence of xylose as an inducer. TT, transcription terminator.

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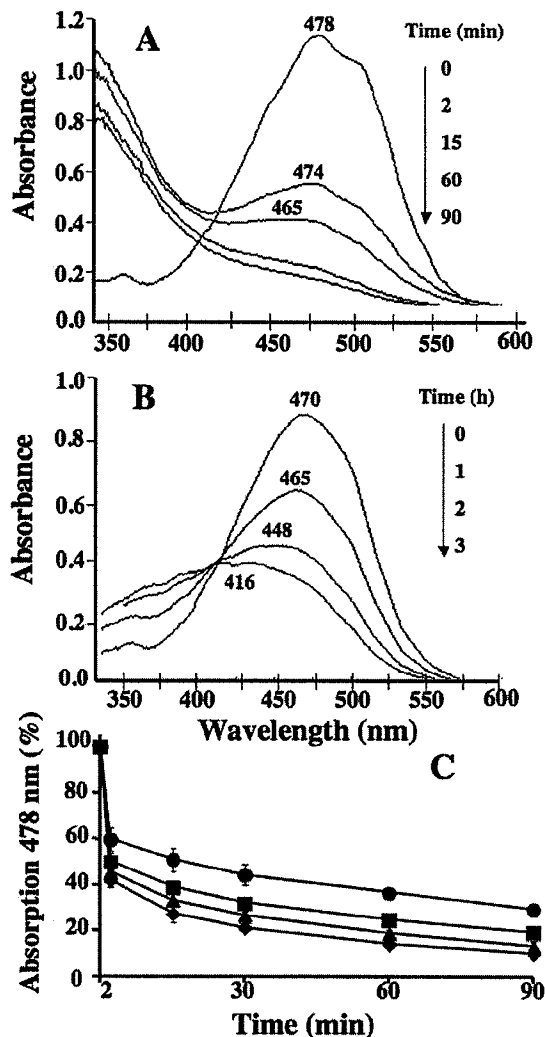

FIG. 2. Time course of staphyloxanthin oxidation by free radicals. (A) Oxidation by free radicals generated in a nonspecific Fenton reaction. The reaction mixture consisted of 12 μM staphyloxanthin in dimethyl sulfoxide-H_2O (4:1, vol/vol), 0.5 mM iron(II) chloride, and 0.5 mM H_2O_2 and was incubated under air at 25°C. Absorption spectra were recorded before the reaction with iron(II) chloride and H_2O_2 (time zero) and after 2, 15, 60, and 90 min. The absorption maxima are indicated. (B) Oxidation by peroxynitrite generated with SIN-1. The reaction mixture consisted of 12 μM staphyloxanthin and 3 mM SIN-1 in ethanol-H_2O (4:1, vol/vol) and was incubated under air at 25°C. Absorption spectra were recorded before reaction with SIN-1 (time zero) and after 1, 2, and 3 h. The absorption maxima are indicated. (C) Time course of oxidation of purified staphyloxanthin by hydroxyl radicals generated in a Fenton reaction. The reaction mixture consisted of 12.5 μM staphyloxanthin in dimethyl sulfoxide-H_2O (4:1, vol/vol) and equimolar concentrations of $FeCl_2$ and H_2O_2, i.e., 0.05 mM (●), 0.1 mM (■), 0.2 mM (▲), and 0.5 mM (◆). The mixture was incubated under air at 25°C. Oxidation of staphyloxanthin was determined by measuring the decrease in absorption at 478 nm. Data points represent the means of five independent experiments. Error bars indicate the deviation of five independent experiments.

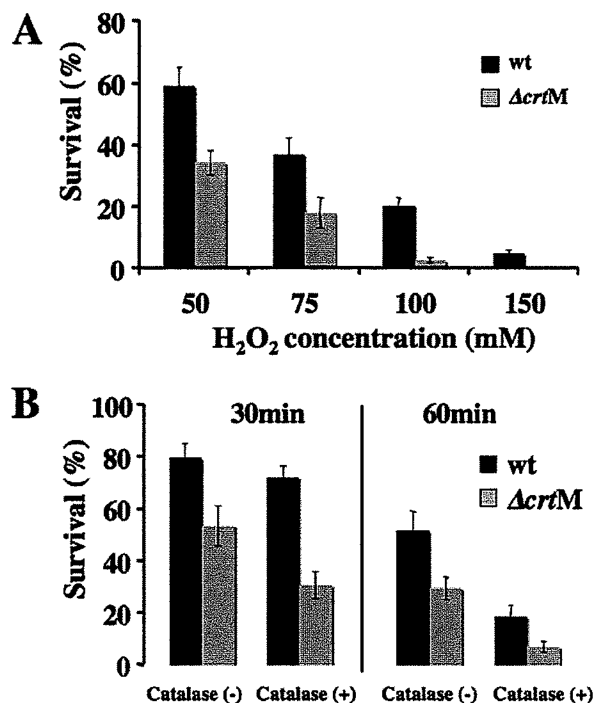

FIG. 3. Effects of H_2O_2 and superoxide radical on the survival of WT and Δ*crtM* mutant *S. aureus* Newman. (A) After 24 h of growth in basic medium, 5 × 10^6 CFU ml^{-1} were incubated in phosphate-buffered saline containing the indicated concentrations of H_2O_2 in the dark at 0°C. After 45 min, the reaction was stopped by destroying the remaining H_2O_2 with 2 U ml^{-1} catalase and incubation for 20 min. Diluted cells (0.1 ml) were spread on BM agar plates. Colonies were counted after 24 h of incubation at 37°C. Values are expressed as a percentage of the CFU in the control culture lacking H_2O_2. Values are the averages of five independent experiments. Error bars indicate the deviation of five independent experiments. (B) After 24 h of growth in basic medium, 5 × 10^6 CFU ml^{-1} were incubated in HEPES buffer containing 10 mM hypoxanthine and 0.1 U of xanthine oxidase (XO) with or without 2 U of catalase at 25°C. After incubation for 30 and 60 min, the reaction was stopped by addition of 10 μM allopurinol. Diluted cells (0.1 ml) were spread on BM agar plates. Colonies were counted after 24 h of incubation at 37°C. Values are expressed as a percentage of the number of CFU in the control culture containing only hypoxanthine (10 mM) and lacking XO. Values represent the average of five independent experiments. Error bars indicate the deviation of five independent experiments.

change. Both *S. aureus* WT and the *crtM* mutant were susceptible to 1.0 mM PMS and succinate (a source of NADH), which generate the superoxide radical O_2^-. Killing correlated with time, but the mutant was more susceptible than the WT (Fig. 4A), which showed twofold higher survival than the mutant. The number of CFU of the control did not change after incubation. Phenazine oxidizes NADH, resulting in production of O_2^-, which can react nonenzymatically with H_2O_2 to form hydroxyl radical (6) and singlet oxygen (10), leading to the peroxidation of polyunsaturated fatty acids of membrane lipids (4, 17).

Myeloperoxidase (MPO) plays a crucial role in bacterial killing by generating hypochlorous acid within neutrophil phagosomes, where it constitutes about 5% of the total neutrophil protein (1, 16). In vitro, MPO in the presence of H_2O_2 and chloride or iodide ions leads to hypochlorous acid and to the killing of many bacteria and fungi (7, 8). We analyzed the sensitivity of WT and *crtM* mutant *S. aureus* to MPO in the presence of H_2O_2 and Cl^-. Both strains were killed, but the *crtM* mutant cells were killed much faster and in greater numbers than WT cells (Fig. 4B). The bactericidal effect was de-

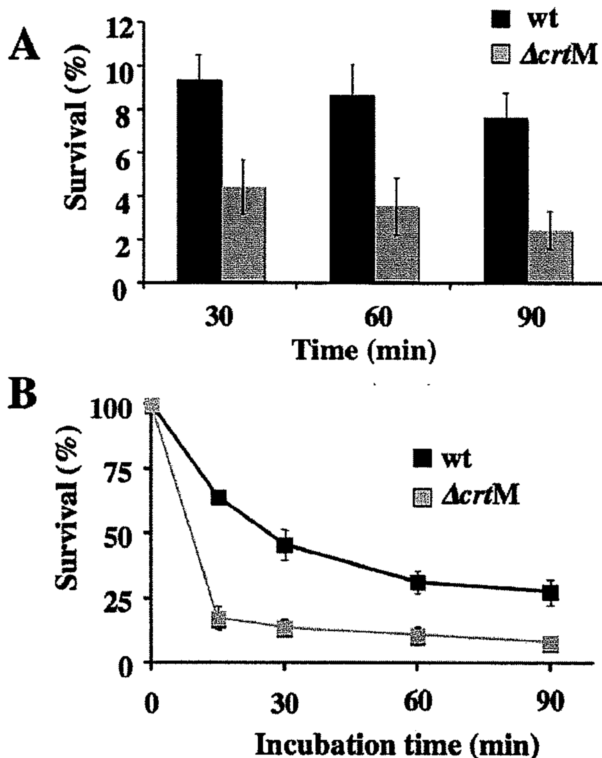

FIG. 4. Effects of PMS and MPO on the survival of WT and Δ*crtM* mutant *S. aureus* Newman. (A) After 24 h of growth in basic medium, cells were harvested and washed twice in HEPES buffer and 5×10^6 CFU ml^{-1} were incubated in 20 mM HEPES buffer containing 1 mM PMS and 2 mM succinate at 25°C. After the indicated time, 0.1 ml of diluted cells was spread on BM agar plates. Colonies were counted after 24 h of incubation at 37°C. Values are expressed as a percentage of the number of CFU in the control culture containing only succinate (2 mM) and lacking PMS. Values are the average of five independent experiments. Error bars indicate the deviation of five independent experiments. (B) After 24 h of growth in basic medium, cells were harvested and washed twice and 5×10^6 CFU ml^{-1} in phosphate-buffered saline at pH 7.4 were mixed with 0.05 U of MPO and 10 μM H_2O_2 and incubated at 25°C for 90 min. Diluted cells (0.1 ml) were spread on BM agar plates. Colonies were counted after 24 h of incubation at 37°C. The number of CFU is expressed as a percentage of the control containing only H_2O_2 (10 μM). Values are the average of five independent experiments. Error bars indicate the deviation of five independent experiments.

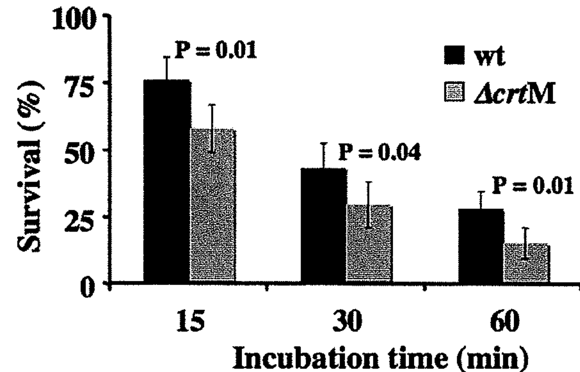

FIG. 5. Killing of WT and Δ*crtM* mutant *S. aureus* Newman by human neutrophils. After 24 h of growth in basic medium, cells were harvested and washed twice in potassium-phosphate buffer (pH 7.2) containing 0.05% human serum albumin. Bacteria (5×10^6 CFU ml^{-1}) were mixed with neutrophils (2.5×10^6/ml). Human serum was added to a final concentration of 10%, and 150 μl of prewarmed Hanks balanced salt solution was also added. Samples (500 μl) were shaken at 37°C, and the incubation was stopped after the indicated time by diluting the samples 100-fold in ice-cold distilled water. The diluted samples (0.1 ml) were spread on BM agar plates, and colonies were counted after 24 h of incubation at 37°C. The number of CFU after incubation with neutrophils is expressed as a percentage of the initial count. Values are the average of five independent experiments. Error bars indicate the deviation of five independent experiments. The significance of experimental differences was evaluated by unpaired Student test.

pendent upon the incubation time, but the overall difference between the strains remained almost constant during the 90 min of incubation; e.g., there was a fourfold difference after 15 min of incubation. The viability of the WT and the *crtM* mutant was not affected by incubation of the cells with NO˙ donors (not shown). NO˙ also has little effect on the viability of other bacterial species, e.g., *Escherichia coli*, *Salmonella* sp., and *Proteus vulgaris* (2, 9).

Finally, we investigated the killing of *S. aureus* by human neutrophils, which consume more O_2 after ingestion of bacteria (15). Since all of the ROS analyzed in this work are also produced during the oxidative burst, it was of interest to compare the killing of the WT and the *crtM* mutant by human neutrophils. Killing of both strains by human neutrophils increased with time, but a higher percentage of the mutant cells were killed (Fig. 5). After 15 and 60 min of incubation, the survival of the WT was 1.3- and 1.8-fold, respectively, higher than that of the *crtM* mutant. Liu et al. (13) described an approximately 10-fold higher survival frequency of the WT compared to the *crtM* mutant in human neutrophils, and they also showed that this effect is not explained by differences in the rate of phagocytosis, because the uptake of WT *S. aureus* was comparable to that of the *crtM* mutant. We saw the same tendency, although the differences between the WT and the *crtM* mutant were less pronounced. One explanation for this discrepancy could be that we used stationary-phase cells throughout our study, where staphyloxanthin production is greatest but where cells might also become more resistant to peroxides and radical species. Other groups have also described better survival of carotenoid-producing cells within human neutrophils (5, 11).

Our data indicate that staphyloxanthin scavenges free radicals with its conjugated double bonds. Since staphyloxanthin is located in the cell membrane, it probably primarily protects lipids but might also be involved in protecting proteins and DNA. Enzymes such as catalase and SOD most likely contribute to a larger extent to the survival of cells during stress and the host response, but staphyloxanthin plays an additional role in the defense against damage by ROS, thereby enhancing the virulence and fitness of the cells. Staphyloxanthin can be regarded as a biological antioxidant against hydrogen peroxide and hydroxyl radicals and might be useful as a therapeutic radical scavenger.

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REFERENCES

1. **Chapman, A. L., M. B. Hampton, R. Senthilmohan, C. C. Winterbourn, and A. J. Kettle.** 2002. Chlorination of bacterial and neutrophil proteins during phagocytosis and killing of *Staphylococcus aureus*. J. Biol. Chem. **277:**9757–9762.
2. **De Groote, M. A., D. Granger, Y. Xu, G. Campbell, R. Prince, and F. C. Fang.** 1995. Genetic and redox determinants of nitric oxide cytotoxicity in a *Salmonella typhimurium* model. Proc. Natl. Acad. Sci. USA **92:**6399–6403.
3. **Duthie, E. S., and L. L. Lorenz.** 1952. Staphylococcal coagulase; mode of action and antigenicity. J. Gen. Microbiol. **6:**95–107.
4. **Fong, K. L., P. B. McCay, J. L. Poyer, B. B. Keele, and H. Misra.** 1973. Evidence that peroxidation of lysosomal membranes is initiated by hydroxyl free radicals produced during flavin enzyme activity. J. Biol. Chem. **248:** 7792–7797.
5. **Fournier, B., and D. J. Philpott.** 2005. Recognition of *Staphylococcus aureus* by the innate immune system. Clin. Microbiol. Rev. **18:**521–540.
6. **Kehrer, J. P.** 2000. The Haber-Weiss reaction and mechanisms of toxicity. Toxicology **149:**43–50.
7. **Klebanoff, S. J.** 2005. Myeloperoxidase: friend and foe. J. Leukoc. Biol. **77:**598–625.
8. **Klebanoff, S. J., A. M. Waltersdorph, and H. Rosen.** 1984. Antimicrobial activity of myeloperoxidase. Methods Enzymol. **105:**399–403.
9. **Klink, M., M. Cedzynski, A. St Swierzko, H. Tchorzewski, and Z. Sulowska.** 2003. Involvement of nitric oxide donor compounds in the bactericidal activity of human neutrophils *in vitro*. J. Med. Microbiol. **52:**303–308.
10. **Koppenol, W. H.** 1976. Reactions involving singlet oxygen and the superoxide anion. Nature **262:**420–421.
11. **Krinsky, N. I.** 1974. Singlet excited oxygen as a mediator of the antibacterial action of leukocytes. Science **186:**363–365.
12. **Lang, S., M. A. Livesley, P. A. Lambert, W. A. Littler, and T. S. Elliott.** 2000. Identification of a novel antigen from *Staphylococcus epidermidis*. FEMS Immunol. Med. Microbiol. **29:**213–220.
13. **Liu, G. Y., A. Essex, J. T. Buchanan, V. Datta, H. M. Hoffman, J. F. Bastian, J. Fierer, and V. Nizet.** 2005. *Staphylococcus aureus* golden pigment impairs neutrophil killing and promotes virulence through its antioxidant activity. J. Exp. Med. **202:**209–215.
14. **Pelz, A., K. P. Wieland, K. Putzbach, P. Hentschel, K. Albert, and F. Götz.** 2005. Structure and biosynthesis of staphyloxanthin from *Staphylococcus aureus*. J. Biol. Chem. **280:**32493–32498.
15. **Sbarra, A. J., and M. L. Karnovsky.** 1959. The biochemical basis of phagocytosis. I. Metabolic changes during the ingestion of particles by polymorphonuclear leukocytes. J. Biol. Chem. **234:**1355–1362.
16. **Segal, A. W.** 2005. How neutrophils kill microbes. Annu. Rev. Immunol. **23:**197–223.
17. **Tappel, A. L.** 1973. Lipid peroxidation damage to cell components. Fed. Proc. **32:**1870–1874.
18. **Wieland, B., C. Feil, E. Gloria-Maercker, G. Thumm, M. Lechner, J. M. Bravo, K. Poralla, and F. Götz.** 1994. Genetic and biochemical analyses of the biosynthesis of the yellow carotenoid 4,4′-diaponeurosporene of *Staphylococcus aureus*. J. Bacteriol. **176:**7719–7726.
19. **Wieland, K. B.** 1999. Organisation und Genexpression der Carotinoid-Biosynthesegene aus *Staphylococcus aureus* Newman und Untersuchungen zur Funktion von Staphyloxanthin. Ph.D. thesis. University of Tübingen, Tübingen, Germany.
20. **Woodall, A. A., S. W. Lee, R. J. Weesie, M. J. Jackson, and G. Britton.** 1997. Oxidation of carotenoids by free radicals: relationship between structure and reactivity. Biochim. Biophys. Acta **1336:**33–42.

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