Derivation of High Enterotoxin B-Producing Mutants of *Staphylococcus aureus* from the Parent Strains

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Certain pH-sensitive (membrane) mutants of *Staphylococcus aureus*, strains 14458 and 778, produce significantly more type-B enterotoxin (SEB) than the parent type. Some carbohydrate mutants (car) from these parent strains also are superior to the parent in SEB formation. By isolating car mutants from high-SEB-producing membrane mutants, it is possible to derive a double mutant producing from six to 50 times as much SEB as the parent type. Inversion of the sequence by isolating pH-sensitive mutants from car mutants does not yield clones with strikingly higher SEB production than the parent strain. The successful isolation sequence (pH-sensitive mutant first and car mutants derived from it) is relatively simple and virtually assures detection of a truly high-SEB-producing clone. The total number of clones whose direct assay for SEB formation is necessary for detection of a high-producing mutant is on the order of 50 to 60.

Detection and isolation of high-enterotoxin-producing mutants of *Staphylococcus aureus* are appreciable aids in the purification and study of these exoproteins. There is currently no known selective medium which permits the growth of high-producing mutants while suppressing background growth of the parent type. Consequently, the few attempts made to isolate such mutants have relied upon assaying, after treatment with mutagen, literally thousands of clones for enterotoxin production. Friedman and Howard (3) were able to increase production of enterotoxin A nearly 20-fold in a stepwise manner. These authors exposed strain 100 to the mutagen, N-methyl-N'-nitro-N-nitrosoguanidine (NTG), and from the survivors were able to detect a mutant which exhibited moderately increased enterotoxin A production. The first step mutant was reexposed to NTG and a mutant with a further increase in enterotoxin A was detected. The procedure was repeated through 13 successive cycles finally yielding mutant 13N-2909 which elaborated much more enterotoxin A than the parent strain 100. A similar procedure, with ultraviolet mutagenesis, had been employed for the derivation of the high-enterotoxin B-producing mutant 10-275 from the parent strain S-6 by Pfizer and Co. In this work, also, a stepwise mutagenesis approach was utilized and required the assay of thousands of clones for enterotoxin B (SEB) production.

This communication describes a relatively simple and comparatively much less-time-consuming method for the derivation of high-SEB-producing mutants from the parent type. For this work *S. aureus* strains 14458 and 778, which are pure SEB producers, were employed (1). The procedure involves mutagenizing the parent strain and selecting membrane mutants as described by Kent and Lennarz (5). An appreciable portion of the membrane mutants can be shown to exhibit increased SEB production (R. A. Altenbern, Can. J. Microbiol., in press). As reported in this paper, some of the NTG-induced mutations to the *car* phenotype (2) also show increased SEB production compared to that of the parent strain. A combination of these two mutations effected by first isolating membrane mutants with higher SEB production and subsequently isolating *car* mutants from the membrane mutants has yielded clones producing 6- to 50-fold greater SEB production than the parent types. The procedure is relatively straightforward and can be completed in a matter of several weeks. In all, less than 60 clones need to be assayed individually.

**MATERIALS AND METHODS**

**Bacterial strains.** *S. aureus*, ATCC 14458, was obtained from stock cultures at this Institute. Strain 778 was kindly furnished by Reginald Bennett of the U.S. Food and Drug Administration. The parent types and all mutants employed were grown on
Production of SEB. Tubes containing 5 ml of liquid NAK medium (7) were inoculated and incubated at an angle on a shaker at 37 C for 24 h. One-tenth milliliter of this culture was added to a 125-ml flask containing 15 ml of NAK medium and the flasks were incubated at 37 C on a reciprocating shaker (85 2-inch [ca. 5.08 cm] strokes per min) for 24 h. Optical densities of 1:5 dilutions of these cultures were determined at 600 nm in a Coleman Jr. spectrophotometer. Five milliliters of the culture were centrifuged; the supernatant fluid was decanted and stored frozen until assayed. The Oudin method for quantitative determination of SEB was employed (9).

Mutagenesis procedure. A tube of 5 ml of YETS medium (8) was inoculated with the desired culture and incubated at an angle on a shaker at 37 C for 24 h. Another tube of fresh YETS broth (5 ml) was inoculated with 0.05 ml of the 24-h culture and was incubated under the same conditions for 6 h. Two-milliliter portions of the 6-h cultures were centrifuged to sediment the cells and the supernatant medium was discarded. The cells were resuspended in 5 ml of filtered sterilized saline containing 200 µg of NTG per ml. After 20 min of exposure to NTG at 37 C, the cells were recovered by centrifugation and resuspended in 5 ml of fresh YETS broth. This culture was allowed to grow overnight at 37 C under the same conditions. These cultures were decimally diluted and plated by spreading 0.05 ml of the dilutions on plates of agar of the desired composition.

Mutant selection. Mutagenized cells, as described above, were plated on Kent and Lennarz agar at pH 7.0 (5). After 24 h of growth at 37 C, single colonies were picked with sterile tooth picks to grid plates of Kent and Lennarz agar at both pH 5.2 and 7.0. After 24 h of incubation, those clones able to grow at pH 7.0 but unable to grow at pH 5.2 were picked to Trypticase soy agar slants for stock cultures. Kent and Lennarz claimed that most of these pH-sensitive clones were membrane mutants by virtue of the drastically altered osmotic stability of the protoplasts or the chemically modified composition of the cytoplasmic membrane (5).

For the detection of carbohydrate mutants (car), mutagenized cells were plated on Korman-Berman agar (6) containing 1% each of lactose, sucrose, and mannitol. Nonfermenting colonies growing on such medium were picked to Trypticase soy agar slants. The nature of the mutants was determined by inoculating these clones into Difco purple broth tubes containing, singly, 1% sugar. The car mutants derived fermented glucose strongly but did not ferment lactose, mannitol, trehalose, levulose, and glycerol. There was feeble fermentation of sucrose at the 1% level by all car mutants but this phenomenon disappeared if the sucrose concentration was decreased to 0.5% or lower. Apparently sucrose fermentation can bypass the components, enzyme I and histidine protein (HPF), of the phosphotransferase system since all these car mutants fermented sucrose strongly at the 5% level. The parent strains fermented all carbohydrates strongly, even at the 0.1% level. Only confirmed car mutants were assessed for SEB production.

RESULTS

SEB production by pH-sensitive (membrane) mutants. Three of the 22 membrane mutants derived from strain 14458 were appreciably better SEB producers. Four of 29 membrane mutants obtained from strain 778 exhibited higher SEB production (Table 1). The remainder of the pH-sensitive clones from both parent strains elaborated either the same as, or less, SEB than the parent type. Under our conditions, strain 778 produced such a small amount of SEB that it was essentially zero by the Oudin assay. The sensitivity limit, in our hands, has been approximately 3 µg per ml. The data expressing micrograms of SEB per milliliter divided by the optical density give some indication of the amount of SEB produced per unit cell mass.

In these experiments, 20 clones, which grew at both pH 7.0 and 5.2 and were not pH-sensitive (membrane) mutants, were picked and evaluated for SEB production. All these clones produced the same amount of toxin as the parent type, indicating that altered SEB production and the pH-sensitive mutation were strongly linked. Many preliminary tests had shown that clones picked at random from nonmutagenized cultures exhibited SEB production within 20% of the parent culture. There is some difference in the amount of SEB produced by strain 14458 and its mutants from several runs (Tables 1 and 2). These discrepancies probably reflect differences in batches of media prepared from new or old lots of the ingredients, NZamine NAK and yeast extract.

| Culture | OD* | µg of SEB/ml | µg of SEB/ml/OD* |
|---------|-----|--------------|------------------|
| 14458 parent | 1.050 | 68 | 65 |
| pH 1 | 0.505 | 98 | 194 |
| pH 5 | 1.050 | 116 | 110 |
| pH 10 | 0.610 | 122 | 200 |
| 778 parent | 0.950 | <3 | 3 |
| pH 6 | 0.800 | 9 | 11 |
| pH 16 | 0.790 | 29 | 36 |
| pH 21 | 0.950 | 47 | 49 |
| pH 24 | 0.860 | 8 | 9 |

* OD, Optical density.
**TABLE 2. SEB production by car mutants derived from S. aureus strains 14458 and 778**

| Culture | OD* | µg of SEB/ml | µg of SEB/ml OD* |
|---------|-----|--------------|-----------------|
| 14458 parent | 0.960 | 105 | 109 |
| car 1 | 0.960 | 149 | 155 |
| car 2 | 0.970 | 149 | 154 |
| car 3 | 0.980 | 149 | 155 |
| car 4 | 0.960 | 185 | 196 |
| car 5 | 0.960 | 199 | 197 |
| car 14 | 0.930 | 147 | 158 |
| car 15 | 0.900 | 107 | 119 |
| car 16 | 0.980 | 163 | 166 |
| 778 parent | 0.980 | <3 | 0 |
| car 1 | 0.830 | 8 | 9 |
| car 2 | 0.880 | 8 | 9 |
| car 5 | 0.870 | 22 | 25 |
| car 6 | 0.960 | 23 | 24 |
| car 3,4,7–11 | 0.900–0.980 | <3 | 0 |

* OD, Optical density.

**SEB production by car mutants.** Of nine *car* mutants isolated from strain 14458, all but one, no. 16, produced significantly more SEB than the parent type. In contrast, only four of 11 *car* mutants derived from strain 778 elaborated elevated levels of SEB (Table 2). From 10 to 12 clones exhibiting the parent fermentation pattern were picked from the mutagenized culture and evaluated for SEB production. All these clones elaborated the same amount of toxin as the parent culture.

**Production of SEB by car mutants derived from selected pH-sensitive mutants of strains 14458 and 778.** *Car* mutants were obtained from the high-toxin-producing, pH-sensitive mutants obtained from strains 14458 and 778 (see Table 1). These double mutants were assessed for SEB production in the hope that some of the clones would display an additive or synergistic effect in regard to the elaboration of toxin. The data presented in Table 3 demonstrate that some clones possessed a remarkably elevated level of toxin production. In particular, *car* 1 and 4 derived from pH mutant no. 5 of 14458 were much better toxin producers. Of the *car* clones isolated from either pH mutant 1 or 10 of 14458, none was appreciably better than the parent pH-sensitive mutant, although this may stem from the comparatively small number of *car* clones assessed. More extensive data on *car* clones isolated from pH-sensitive mutants 16 and 21 obtained from 778 are presented in Table 3.

**TABLE 3. SEB production by car mutants obtained from pH-sensitive mutants 1, 5, and 10 derived from S. aureus strain 14458**

| Culture | OD* | µg of SEB/ml | µg of SEB/ml OD* |
|---------|-----|--------------|-----------------|
| pH 1 parent | 0.630 | 155 | 246 |
| car 1 | 0.670 | 69 | 103 |
| car 2 | 0.740 | 127 | 172 |
| car 3 | 0.710 | 137 | 193 |
| car 4 | 0.620 | 135 | 218 |
| car 5 | 0.600 | 140 | 233 |
| car 6 | 0.540 | 145 | 268 |
| car 7 | 0.590 | 175 | 297 |
| car 8 | 0.575 | 177 | 306 |
| pH 5 parent | 0.890 | 135 | 152 |
| car 1 | 0.740 | 560 | 757 |
| car 4 | 0.860 | 265 | 308 |
| car 5 | 0.840 | 50 | 60 |
| car 7 | 0.790 | 89 | 109 |
| car 8 | 0.800 | 120 | 150 |
| car 16 | 0.840 | 45 | 54 |
| pH 10 parent | 0.580 | 150 | 259 |
| car 1 | 0.640 | 160 | 250 |
| car 2 | 0.565 | 77 | 136 |
| car 3 | 0.565 | 47 | 93 |
| car 8 | 0.670 | 172 | 257 |
| car 9 | 0.495 | 32 | 65 |
| car 10 | 0.530 | 45 | 85 |

* OD, Optical density.

4. *Car* 6 derived from mutant 16 and *car* 10 derived from mutant 21 of 778 showed truly phenomenal increases in SEB production. These data are presented in their entirety to give a picture of the wide variability in SEB elaboration by the individual clones.

**SEB elaboration by pH-sensitive mutants derived from high-producing car mutants of 14458 and 778.** The foregoing data demonstrate well that some *car* mutants derived from high-toxin-producing, pH-sensitive mutants elaborated relatively large amounts of toxin. It might be expected that inversion of the sequence of mutant isolation would be equally effective but such was not the case. Two *car* mutants each from strain 14458 and from strain 778 were mutagenized and pH-sensitive mutants were subsequently isolated. Upon assessment of these double mutants for toxin production, it was found that only an occasional pH-sensitive mutant isolated from the *car* mutant was superior to the parent *car* mutant. The magnitude of the increase was not striking. Therefore, it seems that the order or sequence of isolation of
mutants has a profound influence on probability of detecting a truly high-SEB-producing clone among the mutants obtained.

The condensed data in Table 5 support this conclusion. Only those clones that had retained the car phenotype were assayed for SEB production. About 5% of the pH-sensitive clones exhibited a modified fermentation pattern and were not included in the results in Table 5.

### DISCUSSION

The data presented in this paper stemmed from a general investigation of the effect of certain mutations on production of SEB. Since the pronounced influence of some membrane (pH sensitive) mutations on SEB formation had already been established (R. A. Altenbern, Can. J. Microbiol., in press), efforts were made to assess the effect of other mutations which might induce modifications of the membrane. An attractive possibility in this regard was the phosphotransferase mechanism of concentration and fermentation of sugars, which in some steps intimately involves transport across the cytoplasmic membrane. The initial observation that some car mutants yielded considerably greater amounts of SEB than the parent type prompted the attempts detailed in this work to put both a membrane mutation and a car mutation together in the same cell. Although the car mutation arises from a defect in either enzyme I or HPr of the phosphotransferase system, both of which are apparently cytoplasmic and not membrane bound, it is clear that some car mutations strongly influence SEB formation. It is likely that the transfer of phosphate from phosphorylated HPr to the other components (enzyme II, factor III) of the phosphotransferase system occurs at the cytoplasmic membrane. It is unlikely that SEB is a product of the genes for either enzyme I or HPr since enzyme I has a molecular weight of 100,000 and HPr a weight of 10,000, whereas SEB has a molecular weight of about 28,000. It is not currently known whether the car mutants yielding higher SEB amounts arise from mutations in enzyme I or HPr. An assay system of the extent described by Hengstenberg et al. (4)

### Table 4. SEB production by car mutants derived from pH-sensitive mutants 16 and 21 from S. aureus strain 778

| Culture | OD*  | µg of SEB/ml | µg of SEB/ml |
|---------|------|--------------|--------------|
| pH 16 parent | 0.800 | 29 | 36 |
| car 1 | 0.455 | 0 | 0 |
| car 3 | 0.720 | 21 | 29 |
| car 4 | 0.640 | 54 | 84 |
| car 5 | 0.720 | 21 | 29 |
| car 6 | 0.710 | 158 | 223 |
| car 8 | 0.240 | 0 | 0 |
| car 9 | 0.720 | 13 | 18 |
| car 11 | 0.540 | 0 | 0 |
| car 12 | 0.790 | 22 | 28 |
| car 13 | 0.670 | 67 | 100 |
| car 14 | 0.710 | 0 | 0 |
| car 15 | 0.680 | 22 | 32 |
| car 17 | 0.750 | 0 | 0 |
| car 18 | 0.730 | 46 | 63 |
| pH 21 parent | 0.960 | 31 | 32 |
| car 1 | 0.880 | 13 | 15 |
| car 2 | 0.950 | 24 | 25 |
| car 3 | 0.920 | 59 | 64 |
| car 6 | 0.800 | 13 | 16 |
| car 7 | 0.780 | 24 | 31 |
| car 10 | 0.610 | 230 | 377 |
| car 12 | 0.230 | 0 | 0 |
| car 13 | 0.920 | 0 | 0 |
| car 14 | 0.830 | 24 | 29 |
| car 15 | 0.820 | 33 | 40 |
| car 18 | 0.900 | 22 | 25 |

* OD, Optical density.

### Table 5. SEB production by pH-sensitive mutants isolated from selected car mutants of S. aureus strains 14458 and 778

| Culture | µg of SEB/ml |
|---------|--------------|
| 14458 car 5 | 163 |
| pH 8 | 215 |
| pH 9 | 185 |
| pH 13 | 185 |
| pH 14 | 212 |
| 22 Others | 0-169 |
| 14458 car 14 | 268 |
| pH 11 | 295 |
| 14 Others | 0-238 |
| 778 car 5 | 49 |
| pH 8 | 89 |
| pH 18 | 100 |
| pH 19 | 127 |
| pH 25 | 128 |
| 24 Others | 0-61 |
| 778 car 6 | 30 |
| pH 1 | 57 |
| pH 2 | 55 |
| pH 3 | 57 |
| pH 5 | 72 |
| pH 12 | 55 |
| pH 13 | 47 |
| 12 Others | 0-39 |

* OD, Optical density.
would be required for such analysis. An extensive study of the effect of single sugar fermentation mutations on SEB has been completed and will be published.

The level of SEB formation displayed by the double mutants may be controlled to a considerable extent by the amount of SEB produced by the membrane mutant alone. As examples, pH mutant 5 car 1 (Table 3) produces 6.9 times as much toxin as pH 5 parent alone, whereas pH mutant 21 car 10 (Table 4) produces eight times as much as does its parent, pH 21. These increases are roughly similar in magnitude. By comparison, the 14458 pH 5 mutant (Table 1) secretes 1.7 times the toxin elaborated by the parent, 14458, whereas 778 pH 21 produces about 15 times as much SEB as does the parent strain, 778.

We have shown that starting with a single parent strain (778) a double mutant can be obtained which produces nearly 100-fold greater amounts of toxin than the parent. To date, no double mutant has been isolated which elaborates as much toxin (750 to 1,000 μg/ml) as mutant 10-275 derived from strain S-6 (7). This mutant still reigns supreme in the production of SEB. Unpublished results from this laboratory showed that mutant 10-275 ferments all carbohydrates tested and is no more sensitive to pH than its parent, although protoplasts of 10-275 are more stable than those of S-6. It is likely that strain 10-275 approaches the upper limit for SEB formation.

Recent work in this laboratory (J. F. Metzger, A. D. Johnson, and R. A. Altenbern, unpublished data) has shown that S. aureus strain 494, which produces type D enterotoxin, can be made to increase toxin formation 12-fold by isolating pH-sensitive mutants, as the initial step, and then isolating car mutants from appropriate pH mutants. Other data (R. A. Altenbern, unpublished data) demonstrate that similar treatment of S. aureus strain S-6 can yield mutants with greatly increased type B toxin production. On the basis of these successes, it is suggested that the sequential isolation of pH-sensitive mutants and their car mutants may be a general method of deriving high-enterotoxin-producing clones from parent types of S. aureus.

The observation (R. A. Altenbern, Can. J. Microbiol., in press) that some membrane mutants of strain 14458 display increased alpha-hemolysin production while maintaining SEB production at or below the level of the parent type suggests that there is some specificity of membrane configuration which enhances production of an exoprotein of S. aureus but not of others. It is possible that the sequential isolation of selected pH-sensitive and car mutants may yield clones strikingly superior in formation of one of the other exoproteins elaborated by S. aureus although no efforts are underway in this regard.

The method of isolating high SEB producers here is attractive even though the mechanism for its success remains obscure. The mutation to pH-sensitivity is frequent and comprises 2 to 3% of the survivors from NTG treatment so that many mutants are readily obtained. The car mutation occurs with a frequency of 0.2 to 0.5% in all experiments conducted so far with the NTG treatment detailed in the methods section and thus they can be readily obtained from pH-sensitive mutants. In all, only 50 to 60 clones need to be assayed individually to detect a truly high-toxin-producing mutant. Such a procedure can be of considerable practical value in studies involving purification of such exoproteins and determination of their properties.

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