Lack of Pleiotropic Compensation in Extracellular Protein Production by Hypoproducing Variants of *Staphylococcus simulans* biovar *staphylolyticus*

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The changes in bacterial density, total extracellular protein and activities of three extracellular enzymes were monitored during growth of wild-type *Staphylococcus simulans* biovar *staphylolyticus*, a representative pleiotropic variant that produced decreased levels of the three extracellular enzymes, and a variant that produced only 5 of the 14 extracellular proteins secreted by the wild-type organism. Both variants produced less total extracellular protein than did the parental organism. SDS-PAGE of the proteins secreted by these hypoproducing variants showed that all of the extracellular proteins were produced in decreased amounts. No pleiotropic compensation in extracellular protein production was observed for these hypoproducing variants of *S. simulans* biovar *staphylolyticus*.

INTRODUCTION

Staphylococcus simulans biovar *staphylolyticus* [*S. staphylolyticus* (Sloan *et al.*, 1982)] coordinately produces three extracellular enzymes: a staphylolytic endopeptidase, a micrococcolytic hexosaminidase and a thiol protease (Larrimore *et al.*, 1982; Robinson *et al.*, 1982). Moreover, most mutations affecting the production of any one of these enzymes similarly affect production of the other two as well (Larrimore *et al.*, 1982; Rose *et al.*, 1985).

Björklind & Arvidson (1980) have reported the isolation of mutants of *Staphylococcus aureus* strain V8 with pleiotropic changes in extracellular protein production. They observed a decrease in the production of proteinases I and III, α -toxin, staphylokinase, DNAase and acid phosphatase but an increase in the production of staphylocoagulase and protein A. More recently, Recsei *et al.* (1986) have described a derivative of *S. aureus* strain RN4282 that produced decreased amounts of toxic shock syndrome toxin, staphylokinase and three haemolysins but an increased amount of protein A. Likewise, Coleman (1981) has shown that a low α -toxin-producing variant of *S. aureus* (Wood 46) also produced four other extracellular proteins at reduced levels; however, the differential rates of total extracellular protein formation by the hypoproducing variant and the parental strain were identical. As noted by Coleman (1981), this suggests that other extracellular proteins are produced at higher levels by this variant in order to compensate for decreased production of the five proteins, thereby keeping the extracellular protein-producing capacity saturated. Coleman termed this phenomenon pleiotropic compensation.

To test if pleiotropic compensation occurs in *S. simulans* biovar *staphylolyticus*, we determined the activities of three specific exoenzymes and the concentration of total extracellular protein produced by the wild-type organism, hypoproducers of the three enzymes and a variant that had lost all but 5 of the 14 extracellular proteins produced by the wild-type organism. In addition, the extracellular proteins were separated by SDS-PAGE to determine if there were variations in the levels of production of extracellular proteins other than the endopeptidase, hexosaminidase and protease.

METHODS

Bacterial strains. *Staphylococcus simulans* biovar *staphylolyticus* (NRRL B-2628) was originally obtained from W. C. Haynes (Northern Regional Research Center, Peoria, Ill, USA). Hypoproducing variants for

endopeptidase, hexosaminidase and protease (e.g. strain LP1) were isolated by the procedure described by Larrimore *et al.* (1982). Strain NPR2 is a revertant of a pleiotropic nonproducer of the three exoenzymes; it was identified as endopeptidase-positive and subsequently was shown also to have regained hexosaminidase and protease.

Enzyme assays. Assays for the endopeptidase (Robinson *et al.*, 1979) and for the hexosaminidase and protease (Robinson *et al.*, 1980) have been described previously.

Growth conditions. Cultures were grown in a modification of lysostaphin production medium (Robinson *et al.*, 1980). The modified medium contained (per litre): 60 g enzymic digest of casein (N-Z Amine E, Humko Sheffield Chemical), 5 g NaCl and 2.5 g K_2HPO_4 . For each organism, ten 500 ml Erlenmeyer flasks containing 100 ml of medium were inoculated with 5 ml of an 18 h culture grown in the same medium and were incubated at 37 °C with shaking at 250 r.p.m. Samples (5 ml) were taken at 2 h intervals from 6 to 24 h and the cells were harvested by centrifugation at 12000 g for 10 min at 4 °C. Bacterial density was determined by the procedure of Stormonth & Coleman (1972). Extracellular protein was determined according to the procedure of Bradford (1976).

SDS-PAGE. Samples of supernatants were concentrated 10-fold by ultrafiltration using Millipore immersible CX-10 ultrafiltration units. SDS-PAGE and protein staining were performed according to the procedure of Weber & Osborn (1969) with gels made up to a concentration of 9% (w/v) acrylamide. Samples were loaded onto the gels based on equivalent dry cell weights of the cultures in order to compare the relative amount of each extracellular protein produced per cell. The gels were scanned at 565 nm with a Varian Cary 210 spectrophotometer.

RESULTS AND DISCUSSION

The relationship between growth of wild-type *S. simulans* biovar *staphylolyticus* and secretion of endopeptidase, hexosaminidase, protease and total extracellular protein is shown in Fig. 1(a). *S. simulans* biovar *staphylolyticus* and other staphylococci studied to date characteristically exhibit a biphasic pattern of extracellular protein formation, with increased production in the post-exponential phase of growth (Abbas-Ali & Coleman, 1977; Coleman & Abbas-Ali, 1977; Coleman *et al.*, 1978; Robinson *et al.*, 1980). In modified lysostaphin production medium, *S. simulans* biovar *staphylolyticus* had a 2.6-fold increase in the differential rate of extracellular protein production during post-exponential growth (Fig. 2). A scan of an SDS-polyacrylamide gel of concentrated extracellular proteins from the wild-type organism showed that the organism secreted at least 14 extracellular proteins ranging in molecular mass from 108 to 12.5 kDa (Fig. 3a). The proteins of 38.5, 25.9 and 17.9 kDa correspond to the hexosaminidase, endopeptidase and protease, respectively (H. Mody & G. L. Sloan, unpublished data).

Strain LP1 was typical of several hypoproducing variants that have been studied in this laboratory. These strains were identified on the basis of hypoproduction of the endopeptidase as previously described (Larrimore *et al.*, 1982) and subsequently were found also to be hypoproducers of hexosaminidase and protease. The relationship between growth of strain LP1 and secretion of the three exoenzymes and total extracellular protein is shown in Fig. 1(b). Growth of strain LP1 was quite similar to that of the wild-type organism; however, the levels of production of the three exoenzymes and extracellular protein were significantly less. Strain LP1 also had a biphasic pattern of extracellular protein production (Fig. 2). Whereas the differential rates of extracellular protein production for both the parental organism and strain LP1 were quite similar during exponential growth, the hypoproducer exhibited only a 2.0-fold increase in the differential rate of extracellular protein formation during the post-exponential phase of growth. Therefore strain LP1 was concluded to be deficient in its capacity for post-exponential extracellular protein production. Furthermore, a scan of an SDS-polyacrylamide gel of concentrated extracellular proteins from strain LP1 (Fig. 3b) showed that this variant produced less of each extracellular protein than did the wild-type organism. In fact, the 108 kDa and 102 kDa proteins produced by the parental organism could not be detected among the concentrated extracellular proteins from this hypoproducing organism.

Strain NPR2 is a revertant of a pleiotropic nonproducer of the endopeptidase, hexosaminidase and protease. The pleiotropic nonproducer was isolated as previously described (Larrimore *et al.*, 1982). Strain NPR2 was isolated as an endopeptidase-positive revertant and subsequently was found also to have regained the ability to produce hexosaminidase and protease. The relationship between growth of strain NPR2 and secretion of the three exoenzymes and extracellular protein is shown in Fig. 1(c). Extracellular protein production by

Fig. 1. Relationship between growth and secretion of endopeptidase, hexosaminidase, protease and total extracellular protein. The results shown are representative data from a single experiment; similar results were obtained upon repetition. (a) Wild-type *S. simulans* biovar *staphylolyticus*; (b) strain LP1, a hypoproducer for the three enzymes; (c) strain NPR2, a revertant of a pleiotropic nonproducer of the three enzymes. ○, Bacterial dry weight; ●, extracellular protein; □, endopeptidase; ■, hexosaminidase; △, protease.

this organism also was biphasic (Fig. 2) but with only a 1.8-fold increase in the differential rate of extracellular protein production at the end of the exponential phase of growth. Therefore strain NPR2 was similar to other hypoproducing strains in that it was deficient in its capacity for post-exponential extracellular protein production. In this case, only 5 of the 14 extracellular proteins produced by the wild-type organism could be detected by SDS-PAGE of the concentrated extracellular proteins from strain NPR2 (Fig. 3c). The underlying reason as to why this variant regained the ability to produce only 5 of 14 extracellular proteins is currently under investigation.

In this study we did not observe any pleiotropic compensation in extracellular protein production by hypoproducing variants of *S. simulans* biovar *staphylolyticus* as has been reported for *S. aureus* (Wood 46). Hypoproducing variants for the three extracellular enzymes also were

Fig. 2. Relationship between extracellular protein secretion and growth. The data shown are from the experiment depicted in Fig. 1. ○, Wild-type *S. simulans* biovar *staphylolyticus*; ●, hypoproducing strain LP1; □, revertant NPR2.

Fig. 3. Densitometric scans of SDS-polyacrylamide gels of extracellular proteins produced by: (a) wild-type *S. simulans* biovar *staphylolyticus*; (b) hypoproducing strain LP1; (c) revertant NPR2. Samples were taken at 20 h during the experiment depicted in Fig. 1 and concentrated by ultrafiltration. The gels were loaded with extracellular protein so that each gel contained protein from an equivalent dry weight of cells in order to compare the relative amounts of extracellular proteins produced per cell. The proteins were stained with Coomassie brilliant blue R-250 as indicated in Methods and scanned at 565 nm. The numbers on the peaks indicate molecular masses (kDa).

hypoproducers for all other extracellular proteins secreted by the organism. Furthermore, strain NPR2, which produced detectable levels of only 5 of the 14 extracellular proteins, also produced less total extracellular protein than did the wild-type organism. Engels & Kamps (1982) similarly have shown for two strains of *S. aureus* that staphylocoagulase-negative mutants, which also lost α -haemolysin and PV-leucocidin activity, produced considerably less total extracellular protein than did the wild-type organisms.

Our results and those of Engels & Kamps (1982) indicate that mutations in staphylococci can

cause an overall decrease in the capacity for extracellular protein production. On the other hand, the staphylococcal mutant characterized by Coleman (1981) exhibited decreased production of some extracellular proteins but increased production of others. These results are not necessarily contradictory. The mutants we and Engels & Kamps (1982) have isolated could be deficient in a component required for synthesis or secretion of all extracellular proteins. Such mutants would not be expected to exhibit pleiotropic compensation. However, the mutant isolated by Coleman (1981) could be affected in its ability to synthesize specific subgroups of extracellular proteins. This type of mutant would be expected to show pleiotropic compensation if, for example, availability of ribosomes were the limiting factor for extracellular protein production. Isolation and characterization of additional mutants from *S. simulans* biovar *staphylolyticus* may yet yield variants that show pleiotropic compensation. In any event, the mutants described in this study seem to be specifically defective in protein secretion during the post-exponential phase of growth. Further characterization of such mutants may help to define components involved in extracellular protein production during secondary metabolism.

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