Characteristics of Extracellular Protein Production by *Staphylococcus simulans* biovar *staphylolyticus* during Aerobic and Anaerobic Growth

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Aerobic cultures of *Staphylococcus simulans* biovar *staphylolyticus* characteristically achieved about 17 times higher bacterial densities and produced about 7 times higher concentrations of exoprotein than did anaerobic cultures. However, total exoprotein secreted per unit of bacterial dry weight typically was 2-3 times greater for anaerobic cultures. As determined by SDS-PAGE, anaerobic cultures also produced a wider variety of exoproteins than did aerobic cultures. Three exoenzymes, a staphylolytic endopeptidase, a micrococcolytic hexosaminidase and a thiol protease, were completely repressed during anaerobic growth, which is further evidence for coordination of their production.

INTRODUCTION

Culture supernatants from the stationary phase of aerobic growth of *Staphylococcus simulans* biovar *staphylolyticus* contain at least 14 exoproteins based on SDS-PAGE (Robinson et al., 1987). These include a 25.9 kDa staphylolytic glycylglycine endopeptidase, a 35.9 kDa micrococcolytic endo-β-N-acetylglucosaminidase (hexosaminidase) and a 17.9 kDa thiol protease. Endopeptidase is the active component in lysostaphin, a commercial staphylolytic preparation.

A common control mechanism has been proposed to govern the production of endopeptidase, hexosaminidase and protease by *S. simulans* biovar *staphylolyticus*. Mutants of this organism that have lost endopeptidase activity almost invariably also have lost hexosaminidase and protease activities, and most revertants for one enzyme regain the other two as well (Larrimore et al., 1982; Rose et al., 1985). In addition, endopeptidase, hexosaminidase and protease are coordinately induced by tryptic peptides, coordinately catabolite-repressed by carbohydrates such as glucose, and coordinately relieved of this catabolite repression by derivatives of cyclic AMP (Larrimore et al., 1982; Robinson et al., 1982).

Few studies have been done on the production of exoproteins by staphylococci during anaerobic growth. Coleman et al. (1983) compared the growth and total exoprotein production under aerobic and anaerobic conditions for a number of strains of *Staphylococcus aureus* and found that anaerobic growth resulted in lower cell densities and lower or undetectable exoprotein secretion. Coleman (1985) has also shown that anaerobic cultures of *S. aureus* (Wood 46) have lower bacterial densities and less exoprotein per unit of bacterial dry weight than do aerobic cultures. Furthermore, the pattern of exoproteins produced by anaerobic cultures was also quite different, with α-toxin being the only major exoprotein common to both conditions of growth.

In this study, we report a comparison of the characteristics of exoprotein production during aerobic and anaerobic growth of *S. simulans* biovar *staphylolyticus*.
METHODS

Organism and growth conditions. Staphylococcus simulans biovar staphyloptecus (Sloan et al., 1982) was grown in modified lysostaphin production medium (Robinson et al., 1987). Inocula were prepared by suspending cells grown at 37°C for 48 h on Trypticase Soy Agar (Difco) slants to an OD500 of 0.25 in the growth medium. Portions of these suspensions were added to a final concentration of 1% (v/v) in the growth medium. Aerobic cultures were grown at 37°C in 500 ml Erlenmeyer flasks containing 100 ml of the growth medium with shaking at 250 r.p.m. in a gyratory incubator shaker (model G25, New Brunswick). Anaerobic cultures were grown at 37°C in freshly autoclaved growth medium in 75 ml screw-capped bottles with air excluded. The bottles were incubated in anaerobic jars containing anaerobic indicator strips (GasPak 100 System, Baltimore Biological Laboratory) to verify that anaerobic conditions were maintained. Cells were sedimented by centrifugation at 12000 g for 10 min at 4°C. Bacterial density was determined by the method of Stormorth & Coleman (1972). Extracellular protein was determined by the procedure of Bradford (1976).

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

SDS-PAGE. Culture supernatants were concentrated 20-fold in Amicon Centriprep 10 filters. The concentrated supernatants were subjected to SDS-PAGE according to the procedure of Weber & Osborn (1969), with gels made to a final concentration of 10% (w/v) acrylamide and 0.8% (w/v) N,N'-methylene-bisacrylamide. Samples were loaded onto the gels based on the dry weight of the cells in order to compare the relative amount of each exoprotein produced per cell. The gels were scanned at 565 nm with a Varian Cary 210 spectrophotometer.

Detection of proendopeptidase by trypsin activation. Culture supernatants were reacted with trypsin (20 μg ml⁻¹) to test for the presence of proendopeptidase. The mixtures were allowed to react at 37°C and samples were removed at 10 min intervals for determination of endopeptidase activity.

Preparation of monoclonal antibody and Western blot analysis. ALS8.1, a murine IgG1 monoclonal antibody reactive with endopeptidase, was derived as follows. C3H/HeN mice (6 to 8 weeks old) obtained from the Animal Care Facility of the University of Alabama were immunized by subcutaneous injection of 80 μg lysostaphin (Sigma) emulsified in Freund's complete adjuvant (Difco) followed 7 d later by intraperitoneal injection of 80 μg lysostaphin in phosphate-buffered saline (0.01 M-phosphate buffer, pH 7.0, 0.168 M-NaCl (PBS)). Four days after the final injection, spleen cells from immunized mice were fused to P3X63Ag.8.5 myeloma cells ( Kearney et al., 1979) by the method of Köhler & Milstein (1975). Hybrids were selected in medium containing hypoxanthine, aminopterin and thymidine. Supernatants from resulting outgrowths were screened using an ELISA with lysostaphin as the immobilized antigen. Cells from reactive cultures were cloned by limiting dilution and stable antibody-producing clones were selected.

The monoclonal antibodies were further screened by Western blot analysis for their reactivity with components of lysostaphin and with exoproteins from stationary-phase cultures of S. simulans biovar staphyloptecus. The proteins from both preparations were separated by the method of Laemmli (1970) on a resolving gel of 10% (w/v) acrylamide with a 4% (w/v) stacking gel. The separated proteins were then electrophoretically transferred to a nitrocellulose membrane using a Bio-Rad Trans-Blot cell. To block nonspecific binding, the nitrocellulose blot was treated with a solution of 5% (w/v) powdered skim milk in PBS. The blot was dried and cut into strips and each strip was then reacted with an individual monoclonal-antibody-containing supernatant. After washing, the strips were reacted with peroxidase-labelled goat anti-mouse Ig (Hyclone Laboratories) and were developed in a solution consisting of 60 mg 4-chloro-1-naphthol, 20 ml methanol, 100 ml PBS and 50 μl 30% (v/v) hydrogen peroxide.

Antibody ALS8.1 reacted with a 26 kDa protein in Western blot analysis of lysostaphin and of stationary-phase culture supernatants from S. simulans biovar staphyloptecus. Endopeptidase specificity was confirmed by the ability of ALS8.1 to inhibit endopeptidase activity. ALS8.1 was partially purified from supernatants of cultures producing this antibody by precipitation with 50% saturated ammonium sulphate followed by molecular sieve chromatography using Ultragel Aca 34 (LKB). Purified ALS8.1 was used to detect endopeptidase in similar Western blot analysis of supernatants from aerobically and anaerobically grown cultures of S. simulans biovar staphyloptecus.

RESULTS AND DISCUSSION

The time courses of growth and exoprotein secretion by S. simulans biovar staphyloptecus under aerobic and anaerobic conditions are shown in Fig. 1. As we have reported previously (Robinson et al., 1980, 1987), the majority of extracellular protein produced during aerobic growth was secreted in the post-exponential phase. The relationship between extracellular protein production and growth under aerobic conditions is shown more clearly in Fig. 1(a), where the data have been replotted to show the differential rate of extracellular protein secretion. This rate was biphasic, with the higher rate occurring after the end of the exponential phase of growth. Such a pattern of exoprotein secretion during aerobic growth is characteristic...
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Fig. 1. Time courses of extracellular protein secretion and growth for aerobic and anaerobic cultures of S. simulans biovar staphylolyticus. The results shown are representative data from a single experiment; similar results were obtained upon repetition. ○, Extracellular protein secretion during aerobic growth; ●, extracellular protein secretion during anaerobic growth; □, bacterial dry weight during aerobic growth; ■, bacterial dry weight during anaerobic growth.

Fig. 2. Relationship between extracellular protein secretion and growth for (a) aerobic and (b) anaerobic cultures. The data shown are from the experiment depicted in Fig. 1.

Fig. 1 and Fig. 2 show the dynamics of extracellular protein production and bacterial growth under aerobic and anaerobic conditions. The graphs illustrate that aerobic cultures generally achieve higher bacterial densities and protein concentrations compared to anaerobic cultures.

Of S. simulans biovar staphylolyticus and other staphylococci (Abbas-Ali & Coleman, 1977; Coleman, 1983; Coleman et al., 1978; Robinson et al., 1980, 1987). Although anaerobic growth of S. simulans biovar staphylolyticus resulted in lower bacterial densities, the general pattern of exoprotein secretion was similar to that of aerobically grown organisms (Fig. 1). In particular, the majority of the exoprotein was secreted in the post-exponential phase of growth and the differential rate of exoprotein secretion was biphasic, with the higher rate occurring after the end of the exponential phase (Fig. 2b).

Stationary-phase cultures of S. simulans biovar staphylolyticus in the experiment shown in Fig. 1 had the following densities and exoprotein contents: after aerobic growth (sampled at 20 h), 5.01 mg dry wt ml⁻¹ and 0.150 mg exoprotein ml⁻¹; after anaerobic growth (sampled at 24 h), 0.298 mg dry wt ml⁻¹ and 0.021 mg exoprotein ml⁻¹. Thus the aerobic culture achieved about 17 times higher bacterial density and produced about 7 times higher concentration of total exoprotein than did the anaerobically grown culture. However, total exoprotein secreted per unit of bacterial dry weight was 2.3 times greater for the anaerobically grown culture [0.03 vs 0.07 mg (mg dry wt)⁻¹]. In similar studies on S. aureus (Wood 46) grown in Tryptone Soya Broth, aerobically grown cultures achieved 9 times higher bacterial densities and 12.6 times higher exoprotein concentrations than did anaerobically grown cultures (Coleman, 1985). Thus, in
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Fig. 3. Densitometric scans of SDS-polyacrylamide gels of exoproteins secreted by S. simulans biovar staphylolyticus. At the indicated times during the experiment depicted in Fig. 1, samples were removed and concentrated by ultrafiltration. The gels contained extracellular proteins from equivalent dry weights of cells in order to compare the amounts of each protein produced per cell under different conditions of growth. The proteins were stained with Coomassie brilliant blue R-250 and scanned at 565 nm. The numbers on the peaks indicate molecular masses (kDa). (a) Early stationary phase (20 h) of aerobic growth; (b) early stationary phase (24 h) of anaerobic growth; (c) mid-exponential phase (8 h) of aerobic growth.

contrast to S. simulans biovar staphylolyticus, anaerobically grown S. aureus (Wood 46) produced only 0.7 times as much total exoprotein per unit bacterial dry weight as did aerobically grown cells.

Aerobically grown cultures of S. simulans biovar staphylolyticus produced at least 14 exoproteins, ranging from 108 to 12.5 kDa, as determined by SDS-PAGE of culture supernatants (Fig. 3a). Anaerobically grown cultures produced at least 20 exoproteins, ranging from 108 to 10.7 kDa (Fig. 3b). Based on these exoprotein profiles, as many as 13 of the exoproteins secreted in anaerobic cultures did not correspond to proteins secreted under aerobic conditions. In particular, the supernatants from anaerobically grown cells lacked peaks corresponding to hexosaminidase (38.5 kDa) and endopeptidase (25.9 kDa), but did have a peak (18.1 kDa) that might have corresponded to protease (17.9 kDa). The levels of hexosaminidase, endopeptidase and protease at the beginning of the stationary phase of aerobic growth (20 h) were 2.64, 15.5 and 49.0 units ml⁻¹, respectively. As detectable levels of these three enzymes were not produced during any phase of anaerobic growth, we conclude that the 18.1 kDa protein detected in supernatants from anaerobically grown S. simulans biovar staphylolyticus was not protease. Coleman (1985) also observed quite different patterns of exoproteins for aerobically and anaerobically grown cultures of S. aureus (Wood 46). The only specific protein assayed in his study was α-toxin, which was produced under both growth conditions.

The absence of detectable endopeptidase activity in culture supernatants from anaerobically grown S. simulans biovar staphylolyticus did not preclude the possible presence of an inactive precursor form of this enzyme. Recsei et al. (1987) reported a 64 kDa precursor form of
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Fig. 4. Assay for proendopeptidase in culture supernatants of S. simulans biovar staphylolyticus. Trypsin (20 μg ml⁻¹) was added to each test supernatant and samples were removed at 10 min intervals to assay for endopeptidase activity. The results shown are from a single experiment; similar results were obtained upon repetition. ○, Mid-exponential phase (8 h) of aerobic growth, without trypsin; ●, mid-exponential phase (8 h) of aerobic growth, with trypsin; □, mid-exponential (16 h) or early stationary phase (24 h) of anaerobic growth, with or without trypsin.

Fig. 5. Western blot analysis for detection of endopeptidase and proendopeptidase in culture supernatants. The supernatants were concentrated by ultrafiltration and subjected to SDS-PAGE. The resolved proteins then were transferred to nitrocellulose and reacted with endopeptidase-specific monoclonal antibody. Positions of molecular mass standards are shown at the left. A, Mid-exponential phase (8 h) of aerobic growth; B, early stationary phase (20 h) of aerobic growth; C, mid-exponential phase (16 h) of anaerobic growth; D, early stationary phase (24 h) of anaerobic growth. Bands at 26.0 kDa and 59.0 kDa correspond to endopeptidase and proendopeptidase, respectively.

An endopeptidase that was present in exponential-phase cultures of aerobically grown S. simulans and disappeared concomitantly with the appearance of endopeptidase during the stationary phase. No peak corresponding to a 64 kDa protein was found in the culture supernatant from anaerobically grown stationary-phase cultures of S. simulans biovar staphylolyticus (Fig. 3b). Because Recsei et al. (1987) noted that the proenzyme exhibited aberrant mobility in SDS-PAGE, we examined the exoproteins produced during exponential aerobic growth for a species that was not present in the stationary phase. One peak, due to a 59.0 kDa protein, was present in reduced amounts in the stationary phase with a concomitant increase in the peak due to the mature endopeptidase (cf. Figs 3c and 3a). If the 59.0 kDa protein did correspond to proendopeptidase, then either the 60.5 kDa or the 57.4 kDa protein present in anaerobic culture supernatants could have been the proenzyme (Fig. 3b). This necessitated development of methods that would specifically detect proendopeptidase.

Recsei et al. (1987) concluded that the cleavage site for the conversion of proendopeptidase to mature endopeptidase occurred after a specific arginine residue. This suggests maturation by an
enzyme with trypsin-like specificity. Fig. 4 shows the results from an activation assay utilizing trypsin to convert proendopeptidase to endopeptidase. While this procedure could detect proendopeptidase in exponential-phase aerobic cultures of \textit{S. simulans} biovar \textit{staphylolyticus}, no proendopeptidase was detected in anaerobically grown cultures.

To determine whether the 59-0 kDa protein produced under aerobic conditions was, in fact, proendopeptidase, and to confirm the absence of proendopeptidase from supernatants of anaerobically grown cultures, we reacted Western blots with endopeptidase-specific monoclonal antibody to detect the enzyme and its precursor. The monoclonal antibody reacted with a 26-0 kDa protein (endopeptidase) and a 59-0 kDa protein in an exponential-phase culture supernatant (Fig. 5, lane A). By the stationary phase, the 59-0 kDa protein had disappeared and an increased amount of the 26-0 kDa protein was present (Fig. 5, lane B). These results agree with those of Recsei et al. (1987) regarding maturation of proendopeptidase and thus confirmed that the 59-0 kDa protein was proendopeptidase. These results also showed that a 60-3 kDa protein present in supernatants of stationary-phase aerobically grown cultures (Fig. 3 a) was not residual proendopeptidase. Similar analysis of supernatants from exponential and stationary-phase cultures grown under anaerobic conditions (Fig. 5, lanes C and D) confirmed the absence of both endopeptidase and proendopeptidase in these cultures.

In this study, we have presented further evidence for a common regulatory mechanism controlling production of endopeptidase, hexosaminidase and protease by \textit{S. simulans} biovar \textit{staphylolyticus}, in that production of all three of these exoenzymes was completely repressed under anaerobic conditions of growth. Furthermore, the absence of endopeptidase activity from anaerobic cultures was not due to failure to process proendopeptidase; this precursor was only produced under aerobic growth conditions. However, anaerobic cultures did produce a number of exoproteins that were not present in aerobic cultures. The physiological role of these anaerobically produced exoproteins remains to be determined.

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