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Influence of Growth Temperature and Lipopolysaccharide on Hemolytic Activity of *Serratia marcescens*

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Log-phase cells of *Serratia marcescens* cultured at 30°C were approximately 10-fold more hemolytic than those grown at 37°C. By using a cloned gene fusion of the promoter-proximal part of the hemolysin gene (*shlA*) to the *Escherichia coli* alkaline phosphatase gene (*phoA*), hemolysin gene expression as a function of alkaline phosphatase activity was measured at 30 and 37°C. No difference in alkaline phosphatase activity was observed as a function of growth temperature, although more hemolysin was detectable immunologically in whole-cell extracts of cells grown at 30°C. The influence of temperature was, however, growth phase dependent, because the hemolytic activities of cells cultured to early log phase at 30 and 37°C were comparable. Given the outer membrane location of the hemolysin, lipopolysaccharide (LPS) was examined as a candidate for mediating the temperature effect on hemolytic activity. Silver staining of LPS in polyacrylamide gels revealed a shift towards shorter O-antigen molecules at 37°C relative to 30°C. Moreover, there was less binding of O-antigen-specific bacteriophage to *S. marcescens* with increasing growth temperature, a finding consistent with temperature-mediated changes in LPS structure. Smooth strains of *S. marcescens* were 20- to 30-fold more hemolytic than rough derivatives, a result confirming that changes in LPS structure can influence hemolytic activity. The alkaline phosphatase activity of rough strains harboring the *shlA-phoA* fusion was threefold lower than that of smooth strains harboring the fusion plasmids, a result consistent with a decrease in hemolysin gene expression in rough strains. The absence of a similar effect of temperature on gene expression may be related to less-marked changes in LPS structure as a function of temperature compared with a smooth-to-rough mutational change.

Recently, a hemolytic activity was identified in *Serratia marcescens* (7). In contrast to the majority of bacterial hemolysins described, which are exocellular (1, 26, 30, 31, 37, 40), this activity resided within the cells (7). Two genes, *shlA* and *shlB*, encoding a hemolysin and an accessory protein necessary for activity, respectively (27), are responsible for the hemolytic phenotype of this organism (27). Both gene products are present in the outer membrane (27).

Lysis of erythrocytes by *S. marcescens*, which involves the formation of channels in target membranes (8), resembles lysis by hemolytic *Escherichia coli* (4, 23) and *Aeromonas hydrophila* (15). However, unlike the *E. coli* alpha-hemolysin (29), lysis by *S. marcescens* hemolysin does not require calcium ions (7) and depends on active cellular metabolism (7).

The hemolysin of *S. marcescens* stimulates the release of the inflammatory mediators histamine and leukotrienes in vitro (17), a fact suggesting its role as a pathogenic factor. In addition, iron regulation of hemolysin gene expression has recently been demonstrated (K. Poole and V. Braun, Infect. Immun., in press). This finding, together with the ability of hemolysins to release heme iron-containing hemoglobin, implies that hemolysin may aid iron acquisition, as well. This role would be especially important given the low levels of available iron in the host resulting from the presence of the iron-binding proteins transferrin and lactoferrin (9).

In the present study, we examined the influence of temperature on the hemolytic activity of *S. marcescens*. Growth temperature has been implicated as a factor regulating the expression of virulence factors in *Yersinia pestis* (12), *Shigella* sp. (21), and enterohemorrhagic *E. coli* (36), and exocellular nuclease production by *S. marcescens* is similarly influenced by growth temperature (16). We report that temperature, indeed, influences hemolytic activity, although apparently via alterations in lipopolysaccharide (LPS) structure, which we demonstrated to have marked effects on hemolytic activity.

MATERIALS AND METHODS

Bacterial strains, bacteriophage, and growth conditions. The bacterial strains used in this study are listed in Table 1. YR-B and YR-C are LPS-specific *S. marcescens* phages (Y. Ruan, M.S. thesis, University of Tübingen, Federal Republic of Germany, 1987). L broth (1% [wt/vol] tryptone–0.5% [wt/vol] yeast extract–0.25% [wt/vol] NaCl) was the rich medium used throughout. M9 glucose (20) was the minimal medium used. Tetracycline (10 μg/ml) and kanamycin (25 μg/ml) were included in the growth media as required. Cells were cultured aerobically at 30 or 37°C.

Plasmids. Plasmid pKP100 was a pLG339 (32) derivative carrying the hemolysin operon (5′-*shlB-shlA*-3′) of *S. marcescens* with an in-frame fusion of the *E. coli* alkaline phosphatase *phoA* gene to the promoter-proximal portion of *shlA*, the hemolysin gene (Poole and Braun, submitted). Plasmid pKP101 was a pLG339 derivative carrying the fusion described above but lacking most of the 5′ noncoding sequences upstream of the hemolysin genes (Poole and Braun, submitted). Plasmid pKP102 was a pLG339 derivative carrying the intact hemolysin determinant of *S. marcescens* (Poole and Braun, submitted).

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TABLE 1. Bacterial strains used

Strain	Description	Source or reference
S. marcescens		
W1128	Wild-type human isolate	7
2055	LPS-deficient derivative of strain W1128; selected as resistant to phage YR-C	Y. Ruan
W1436	Exoprotein-deficient derivative of strain W1128	8
1765	Rough derivative of strain W1436	K. Hantke
1763	Spontaneous smooth revertant of strain 1765	K. Hantke
E. coli F860	09:K29$^-$:H$^-$ *his pmi*	13

DNA methods. Plasmid DNA was isolated by the alkaline lysis procedure (5). For the quantitative recovery of plasmid DNA from *S. marcescens*, cells were first heated at 65°C for 2.5 min at an A_{578} of 6.0 in 10 mM NaCl and washed twice with 10 mM NaCl as a precaution against an endogenous nuclease activity present in this organism (11, 33). Plasmid DNA isolated from *E. coli* and possessing *Sma*I restriction sites was treated with *Hpa*II methylase in vitro prior to use in the transformation of *S. marcescens* (2). DNA-modifying enzymes were used per manufacturer instructions. The transformations of *E. coli* (20) and *S. marcescens* (28) have been described previously.

Assays. The alkaline phosphatase activity of bacterial cells harboring in-frame *phoA* fusions to the cloned hemolysin genes was determined as described previously (24) with modifications (Poole and Braun, submitted). The hemolysis assay has been described elsewhere (7).

Cell fractionation and SDS-polyacrylamide gel electrophoresis. Outer membranes were prepared from isolated cell envelopes via differential solubility in Triton X-100 (27). The preparation of whole-cell protein extracts has been described elsewhere (25). LPS was prepared from whole-cell lysates following proteinase K digestion (14) and visualized in polyacrylamide gels by using the silver stain procedure of Tsai and Frasch (35). Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was performed by the method of Lugtenberg et al. (18) with 7 or 11% (wt/vol) acrylamide in the running gel.

Antisera and immunoblotting. The preparation of antiserum to the ShlA and ShlB polypeptides has been described previously (27). Western immunoblotting of proteins separated on SDS-polyacrylamide gels was performed by the method of Towbin et al. (34) with modifications (27).

Phage-binding assays. Bacterial cells were harvested and suspended in 5 mM $CaCl_2$–50 mM $MgSO_4$. Bacteria (4×10^8 CFU) and phage (4×10^6 PFU) were incubated at 30°C for 25 min, the bacteria and bound phage were removed by centrifugation, and the titers of unbound phage were determined at 30°C on strain W1128.

RESULTS

Temperature effects on hemolytic activity. To assess the influence of growth temperature on the hemolytic activity of *S. marcescens*, cells were cultured at 30 or 37°C and harvested at log phase prior to assay for hemolysis. Hemolytic activity decreased with increasing growth temperature. After 30 min of incubation with the erythrocytes, bacterial cells cultured at 37°C gave 10-fold less lysis compared with those grown at 30°C (Fig. 1). Western immunoblots of whole-cell extracts (Fig. 2A) confirmed the presence of less hemolysin in cells grown at 37°C compared with those grown at 30°C. Interestingly, the levels of the outer membrane protein ShlB also declined at 37°C (Fig. 2B).

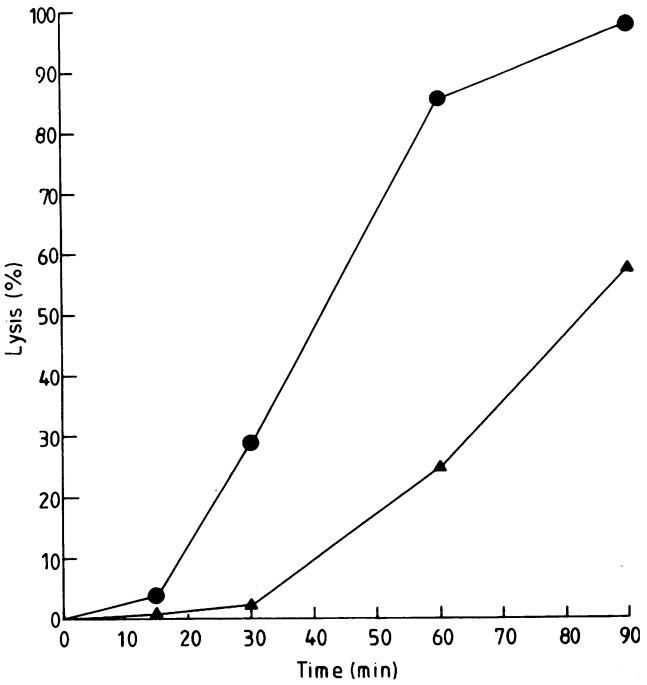

FIG. 1. Time-dependent lysis of erythrocytes incubated with cells of *S. marcescens* W1128 cultured at 30 (●) or 37°C (▲). Log-phase cells were harvested, suspended in NaCl (0.9%, wt/vol) to an A_{578} of 0.3, and incubated at 30°C with an equal volume of erythrocytes (16%, vol/vol).

The hemolysin determinant of *S. marcescens* comprises two adjacent genes, *5'-shlB-shlA-3'*, with the major promoter for hemolysin gene (*shlA*) expression present upstream of *shlB*. To determine whether the influence of temperature on hemolytic activity occurred at the level of transcription, plasmid pKP100, bearing a gene fusion between the 5' end of *shlA* and the *E. coli* alkaline phosphatase gene *phoA*, was introduced in *S. marcescens*, and hemolysin gene expression was monitored as a function of alkaline phosphatase activity. No differences in alkaline phosphatase activity were observed at temperatures between 30 and 37°C (data not shown).

The increase in hemolytic activity at 30°C was observed for log-phase cells. To characterize this effect in more detail, the influence of temperature was examined throughout the growth cycle (Fig. 3). When cells grown at 30 and 37°C were compared, it was noted that early-log-phase cells (A_{578} = 0.2) exhibited strikingly similar hemolytic activities that diverged with continued growth. Cells cultured at 30°C demonstrated high levels of hemolytic activity throughout the logarithmic phase of growth, which declined noticeably only when A_{578} values exceeded 1.0. In contrast, the hemolytic activity of cells grown at 37°C declined sharply from a maximum at A_{578} = 0.2, with little activity detectable at cell densities above A_{578} = 0.6.

Temperature-mediated LPS changes. There were no temperature effects on hemolysin for *E. coli* K-12 derivatives

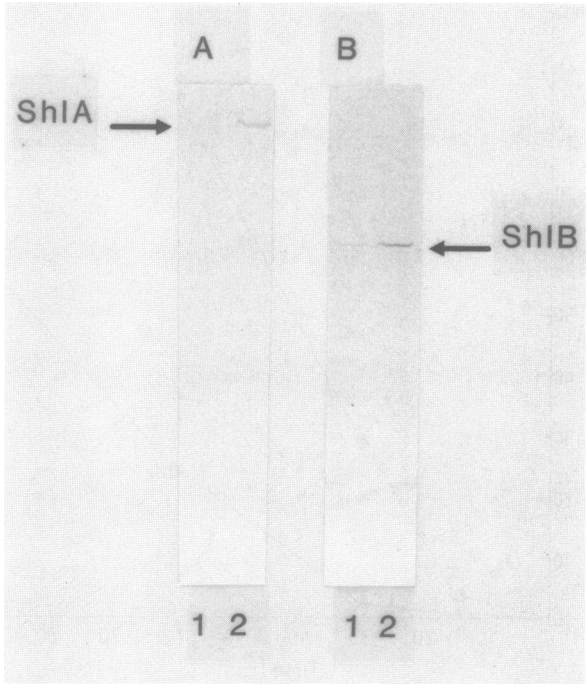

FIG. 2. Immunodetection of hemolysin, ShlA (A), and ShlB (B) in Western blots. Electrophoretically separated whole-cell protein extracts (100 μg) (A) or outer membrane proteins (40 μg) (B) of log-phase W1128 cells cultured at 37 (lanes 1) or 30°C (lanes 2) were transferred to nitrocellulose and probed with antiserum to ShlA (A) or ShlB (B).

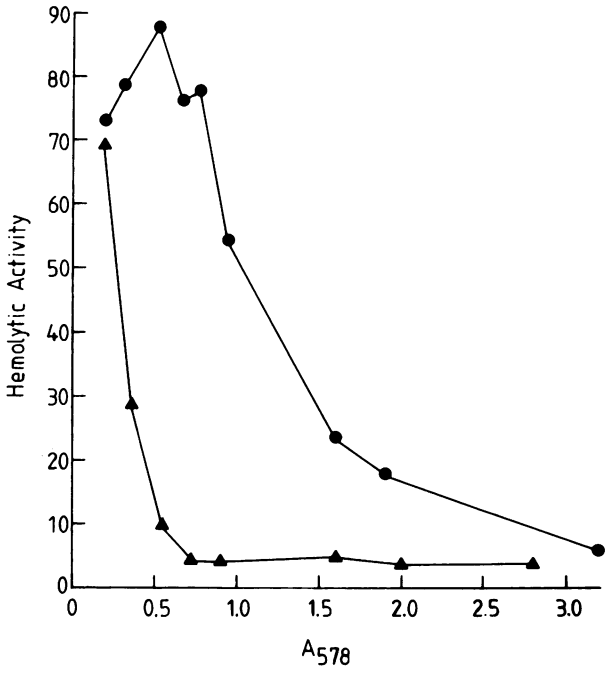

FIG. 3. Hemolytic activity of *S. marcescens* W1128 as a function of growth at 30 (●) or 37°C (▲). Growing cells harvested at the indicated cell densities (A_{578}) were prepared and assayed for hemolytic activity as described in the legend to Fig. 1. Hemolytic activity is reported as the percentage of erythrocytes lysed after 30 min of incubation with bacterial cells.

harboring the cloned hemolysin operon (data not shown), a result ruling out a direct effect of temperature on hemolysin activity. The observation that cells grown at 30 and 37°C exhibited comparable hemolytic activities at early log phase further supports the absence of a direct temperature effect on hemolysin activity. Thus, the temperature effect is probably mediated by another component in *S. marcescens* that interacts with the hemolysin.

Both hemolysin proteins are located in the outer membrane (27), so that some interaction with LPS is probably inevitable. Furthermore, changes in LPS as a function of temperature have been reported for *Salmonella anatum* (22) and *Pseudomonas aeruginosa* (10). Thus, we examined LPS for temperature-related alterations that might mediate this effect on hemolytic activity. Silver staining of LPS prepared from cells grown at 30 and 30°C revealed an apparent increase in molecules with shorter O-antigen chain length in cells grown at 37°C (Fig. 4), with a concomitant decrease in molecules with longer chain length (compare the staining intensities of the two largest LPS species in Fig. 4, lanes 1 and 2).

To further assess changes in LPS structure that might be induced by changes in temperature, we examined the efficiency with which O-antigen-specific phages plated on cells at 30 and 37°C. Unfortunately, although both phages plated on *S. marcescens* at 30°C, they failed to form plaques at 37°C. To overcome the apparent temperature sensitivity of the phages, bacterial cells were cultured at both temperatures to late log phase (A_{578}, 0.7 to 0.8), when temperature-mediated differences in hemolytic activity were maximal (Fig. 3), and examined for their ability to adsorb phage. More phages (5- to 10-fold) remained unbound following

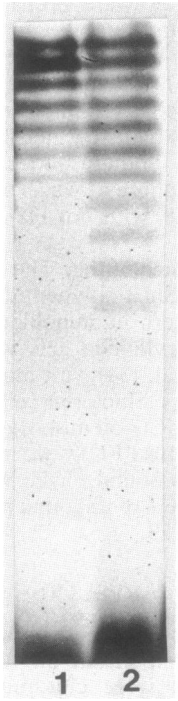

FIG. 4. Silver-stained SDS-polyacrylamide gel electrophoretogram of LPS prepared from 3×10^7 *S. marcescens* W1128 cells cultured at 30 (lane 1) or 37°C (lane 2).

TABLE 2. Adsorption of O-antigen-specific phage by *S. marcescens* W1128[a]

Phage	Bacterial growth temp (°C)	Amt of unbound phage[b] (PFU [10^3])
YR-B	30	1.5
	37	14.1
YR-C	30	7.9
	37	34.8

[a] Bacteria (4×10^8 CFU) and phage (4×10^6 PFU) were incubated at 30°C, and the titer of unbound phage was determined after removal of bacterial cells and bound phage by centrifugation.
[b] Values are representative of three to five experiments.

incubation with cells grown at 37°C compared with incubation with cells grown at 30°C (Table 2). In contrast, early-log-phase cells showed only a marginal (twofold) difference in the number of phage left unbound as a function of growth temperature (data not shown). Apparently, the adsorption of phages YR-B and YR-C strongly depend on the density of long O-antigen chains at the cell surface.

LPS effects on hemolytic activity. To assess directly any influence LPS might have on hemolytic activity, we compared the hemolytic activity (Table 3) of smooth strains (Fig. 5, lanes 1, 3, and 5) with O-antigen-deficient (Fig. 5, lane 2) and rough (Fig. 5, lane 4) derivatives of these strains. Without exception, smooth strains were markedly more hemolytic, exhibiting 20- to 30-fold more activity than the corresponding rough derivatives (Table 3). Indeed, spontaneous reversion of strain 1675 to the smooth phenotype (strain 1673; Fig. 5, lane 5) restored the hemolytic activity to the level observed for the smooth parent strain of this rough derivative (Table 3). Consistent with these data, we observed higher levels of hemolysin in whole-cell extracts of smooth strains compared with those of rough strains (Fig. 6A).

LPS effects on hemolysin gene expression. Using the *shlA-phoA* fusion vector pKP100, we examined the influence of LPS phenotype on hemolysin gene expression as a function of alkaline phosphatase activity. Rough and O-antigen-deficient strains exhibited threefold-less alkaline phosphatase activity compared with their smooth counterparts (Table 4), a result consistent with the reduced expression of the hemolysin gene in these strains.

We previously demonstrated that the major promoter for hemolysin gene expression lies upstream of the 5′-proximal *shlB* gene. Deletion of promoter sequences upstream of *shlB* in a *shlA-phoA* fusion vector resulted in a 10-fold decrease in

TABLE 3. Hemolytic activity of smooth and rough strains of *S. marcescens*

Strain	LPS phenotype[a]	Hemolytic activity[b] (% lysis)
W1128	Smooth	74.7
2055	O antigen deficient	2.7
W1436	Smooth	68.5
1765	Rough	3.5
1763	Smooth	86.7

[a] Phenotypes were deduced from silver staining of LPS preparations (Fig. 5).
[b] Hemolytic activity is expressed as the percentage of erythrocytes lysed after 30 min of incubation with bacterial cells at 30°C. Data were derived from experiments performed as described in the legend to Fig. 1. Values are representative of three experiments.

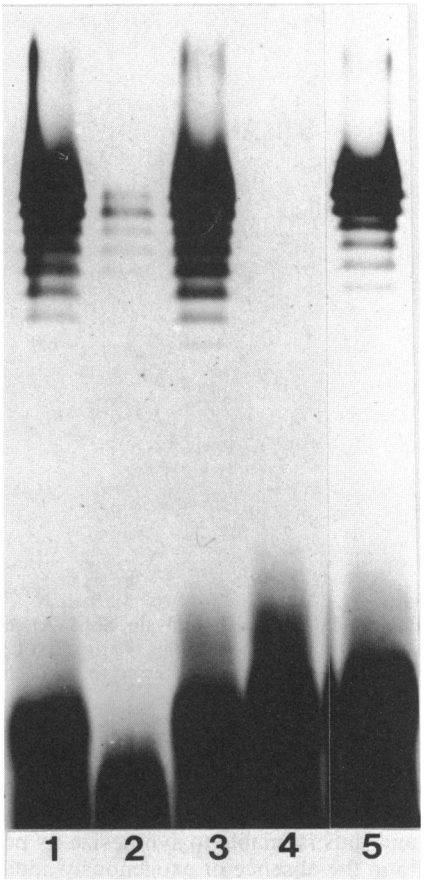

FIG. 5. Silver-stained SDS-polyacrylamide gel electrophoretogram of LPS prepared from 2×10^8 cells of *S. marcescens* W1128 (lane 1), 2055 (lane 2), W1436 (lane 3), 1675 (lane 4), and 1673 (lane 5) cultured at 30°C.

alkaline phosphatase activity (Poole and Braun, submitted). We have also localized sequences required for mediating the iron regulation of hemolysin gene expression to this upstream region (Poole and Braun, submitted). To determine whether the influence of LPS on hemolysin gene expression was similarly mediated by sequences upstream of *shlB*, we deleted this upstream region in the *shlA-phoA* fusion vector. The resultant vector, pKP101, was then introduced into the various strains, and alkaline phosphatase activities were determined. Both smooth and rough strains harboring pKP101 showed marked decreases (2.5- to 9-fold) in alkaline phosphatase activity compared with the same strains carrying pKP100 (Table 4). Furthermore, the residual activity present in strains carrying pKP101 was strikingly constant irrespective of LPS phenotype. These data suggested that the LPS effect on hemolysin gene expression was, indeed, mediated at a site upstream of *shlB*. Consistent with this, we also observed LPS-specific effects on ShlB expression. As with hemolysin, outer membrane levels of ShlB were reduced in rough O-antigen-deficient strains (Fig. 6B).

Specificity of LPS requirement. The apparent requirement for O side chains for maximum hemolysin protein levels and hemolytic activity might be a general one in that the structure of a rough outer membrane will be substantially altered relative to a smooth outer membrane (19). On the other hand, hemolysin may interact in a very specific manner with *S. marcescens* O-side-chain molecules. To distinguish be-

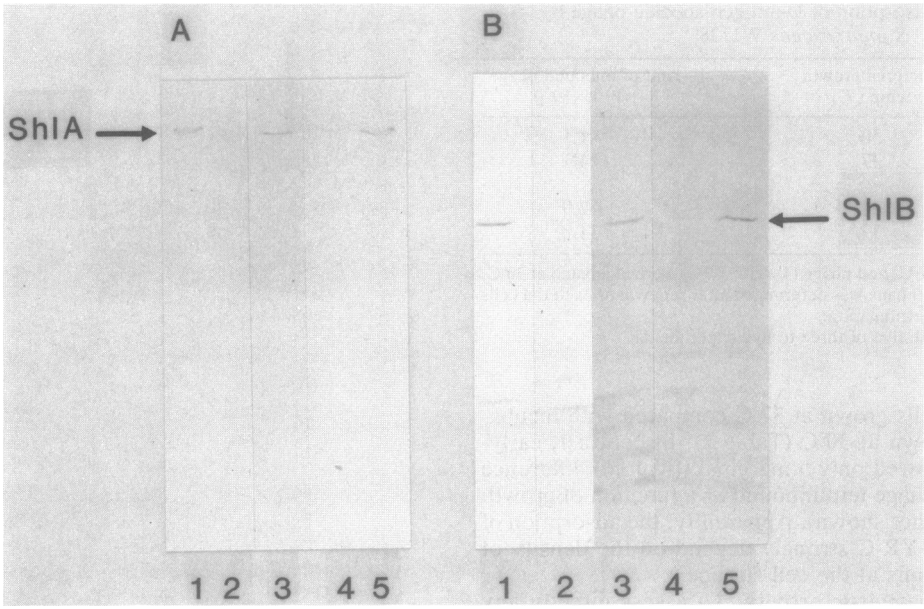

FIG. 6. Immunodetection of hemolysin, ShlA (A), and ShlB (B) in Western blots. Electrophoretically separated whole-cell protein extracts (100 μg) (A) or outer membrane proteins (40 μg) (B) of 30°C grown log-phase W1128 cells of strain (lane 1), 2055 (lane 2), W1436 (lane 3), 1675 (lane 4), and 1673 (lane 5) were transferred to nitrocellulose and probed with antiserum to ShlA (A) or ShlB (B).

tween these possibilities, we introduced the hemolysin genes into *E. coli* F860 *pmi* on a medium-copy-number vector (pKP102). Strain F860 lacks a functional phosphomannose isomerase and thus is unable to synthesize its polymannose O side chain in the absence of exogenously added mannose (13). By culturing *E. coli* F860 (pKP102) in the absence or presence of mannose, we could, in general, compare the influence on hemolytic activity of a rough (Fig. 7 inset, lane 1) and a smooth (Fig. 7 inset, lane 2) background, respectively. Hemolytic activity increased twofold in O-side-chain-producing cells grown in the presence of mannose (Fig. 7). Still, this increase in activity did not approach the 20- to 30-fold increase seen in smooth strains of *S. marcescens* compared with rough strains.

TABLE 4. Alkaline phosphatase activity of *S. marcescens* strains harboring *shlA-phoA* fusion vectors[a]

Strain	LPS phenotype[b]	APase activity (10^3)[c]	
		pKP100	pKP101
W1128	Smooth	37.7	4.0
2055	O antigen deficient	12.3	4.3
W1436	Smooth	37.3	4.5
1765	Rough	13.3	4.0
1763	Smooth	39.8	4.3

[a] Stationary-phase L broth cultures of strains carrying the indicated *phoA* fusion vectors were diluted into fresh L broth containing kanamycin and grown to log phase. Cells were harvested, and the alkaline phosphatase activity of permeabilized cells was determined. To confirm that plasmids did not exhibit any strain-dependent variation in copy number, plasmid DNA was prepared from all samples and quantitated visually on ethidium bromide-stained agarose gels. No alkaline phosphatase activity was detectable in culture supernatants.

[b] Deduced from silver staining of LPS (Fig. 5).

[c] Alkaline phosphatase (APase) activity is reported as ΔA_{405} per minute per A_{578}. All values were adjusted for the background activity of the chromosomally encoded alkaline phosphatase present in these strains. Background levels were typically 2.0. Values are representative of two to four experiments. In pKP101, the promoter region upstream of *shlB* was deleted.

DISCUSSION

In the present study we found that the hemolytic activity in *S. marcescens* was inversely related to the growth temperature. Indeed, we noted that for cells cultured at temper-

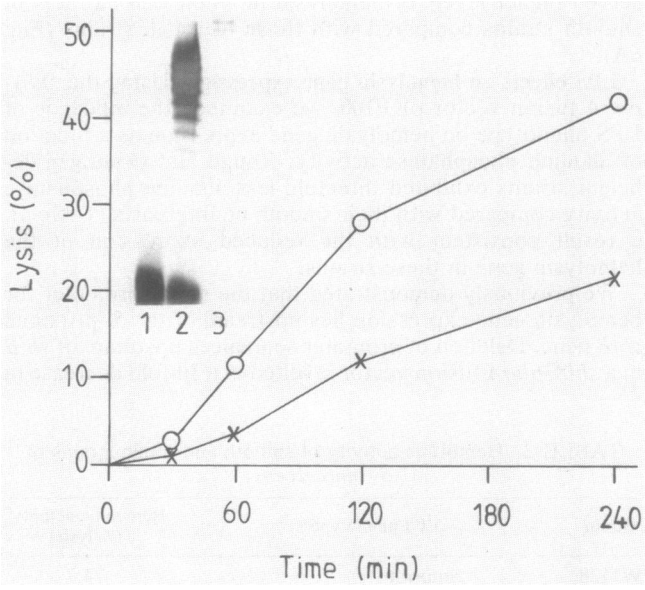

FIG. 7. Time-dependent lysis of erythrocytes incubated with *E. coli* F860(pKP102) grown in the presence (○) or absence (×) of mannose. Stationary-phase M9 glucose cultures of strain F860 were diluted into fresh M9 glucose with or without 0.2% (wt/vol) mannose and grown to log phase at 30°C. Cells were harvested and assayed for hemolytic activity as described in the legend to Fig. 1. (Inset) Silver-stained LPS prepared from *E. coli* F860(pKP102) grown in the absence (lane 1) or presence (lane 2) of mannose. Lane 3, LPS prepared from *S. marcescens* W1128. Lane 3 was underloaded to highlight the major LPS species present in this strain.

atures as low as 22°C, hemolytic activity still increased with decreasing growth temperature (K. Poole, unpublished result). Interestingly, this effect was not mediated at the level of gene expression, although whole-cell hemolysin levels did correlate with activity. The observed temperature-mediated changes in LPS structure could, however, account for the temperature effects on hemolytic activity, in view of the influence of mutational changes in LPS structure on activity. Certainly, the apparently less-marked influence of temperature on LPS structure, compared with the effect of smooth-to-rough mutational changes, correlated with the less-drastic effects of temperature on hemolytic activity.

The role of LPS in the hemolytic activity of *S. marcescens* remains to be elucidated. One possibility is that LPS interacts with the hemolysin to stabilize the protein. Alterations in LPS structure, mediated by temperature or by mutation, may affect such an interaction and hence influence hemolysin stability. This possibility would explain our observation that hemolysin levels decline at 37°C with no concomitant decrease in gene expression. Whether LPS is also an essential component of a hemolytic complex remains to be seen. Certainly the alpha-hemolysin of *E. coli*, which, like the *S. marcescens* hemolysin, apparently lyses erythrocytes via formation of channels in target membranes (4, 8, 23), exists as a complex which includes LPS (6). Additionally, an LPS-hemolysin interaction may be important for the export to and correct insertion of hemolysin into the outer membrane. Again, interference with this process could affect the recovery of hemolysin. Interestingly, ShlB levels also decreased with an increase in growth temperature and in rough mutants, a result suggesting that ShlB, too, depends to some extent on LPS for proper insertion into the outer membrane or maintenance.

The shift towards shorter chain length and unsubstituted rough-core LPS with increasing growth temperature contrasts with results obtained for *S. anatum* (22) and *P. aeruginosa* (10), for which a decrease in the level of rough-core substitution by O-antigen molecules was observed at lower temperatures. Indeed, binding of an LPS-specific phage to *S. anatum* increased with increasing growth temperature, whereas we noted that the binding of LPS-specific phages YR-B and YR-C to *S. marcescens* decreased with increasing growth temperature. Apparently, the enzymes involved in LPS biosynthesis in these organisms are differentially influenced by temperature. Still, the decrease in hemolytic activity with a decline in O-antigen levels or a decrease in mean O-side-chain length implies a requirement not only for O-side-chain molecules for hemolytic activity but perhaps for O-side-chain molecules of a defined length. In this regard, it was interesting that there was only a slight increase in the hemolytic activity of *E. coli* F860 carrying the cloned hemolysin genes upon synthesis of O side chain compared with the activity of the same strain lacking O-side-chain molecules, because the longest O-side-chain molecules in this strain were still shorter than the major LPS species in *S. marcescens* (Fig. 7, lanes 2 and 3). Furthermore, the decrease in number of LPS molecules in *S. marcescens* at 37°C was concomitant with a decrease in hemolytic activity. One cannot, however, rule out the possibility that hemolysin interacts specifically with *S. marcescens* LPS and that other LPS species are inappropriate for this interaction. The OmpA products of the cloned *ompA* genes from a number of clinical *E. coli* isolates are, for example, poorly expressed in *E. coli* K-12 unless the genes are mutagenized in such a way that the resultant OmpA products demonstrate an enhanced ability to interact with K-12 LPS (3).

Lipase activity (39) and synthesis of the pigment prodigiosin (unpublished observation) also decrease in rough strains of *S. marçescens*. Furthermore, a decrease in exocellular nuclease (16) and prodigiosin (38) production by *S. marcescens* has been correlated with an increase in the growth temperature from 30 to 37°C, parallel with alterations in LPS structure (this study). Apparently, the production or activity of a number of component molecules is influenced by LPS structure in this organism.

Deep rough mutants of *Salmonella typhimurium* and *E. coli* exhibit a deficiency in a number of outer membrane proteins (19). However, we observed no such general deficiency in outer membrane proteins in the rough strains described here, a fact indicating a specific influence of LPS changes on hemolysin (ShlA) and ShlB levels in *S. marcescens*. Given the magnitude (30-fold) of the influence of a mutational smooth-to-rough transition on hemolytic activity, which correlated at least qualitatively with the detected levels of the hemolysin proteins, it is, perhaps, not surprising that expression of the hemolysin operon also apparently decreased in rough strains. The absence of a similar effect of temperature on gene expression probably reflects the less-marked changes in LPS structure observed as a function of temperature. The decrease in hemolysin gene expression in a rough background is undoubtedly a response to an environment that is less than optimal for the maintenance of hemolysin proteins. Because less of the hemolysin proteins can be functional in rough strains, it makes sense for the cell to decrease the expression of these proteins.

One might argue that changes in LPS structure might influence the stability and, hence, activity of the ShlA-PhoA fusion protein used to monitor the expression of the hemolysin operon in this study, especially in view of the demonstrated influence of LPS structure on hemolysin. Although this influence on the activity of the ShlA-PhoA fusion protein could account for a decrease in alkaline phosphatase activity in rough strains, in the absence of any effects on gene expression, it is probably not the case. The fusion used in this study possessed perhaps 50 N-terminal amino acid residues of ShlA, including 30 amino acids which compose the signal peptide of this protein (27) and which will be cleaved upon export of the fusion protein. Because of the limited amount of ShlA present, the potential for ShlA-mediated interaction of the fusion protein with LPS is probably minimal. Furthermore, the fusion protein is, indeed, quite unstable, being degraded, however, to an enzymatically active, stable product that is indistinguishable from the native alkaline phosphatase in size and that probably accounts for the enzyme activity measured. In the absence of ShlA sequences, any discussion of LPS-mediated influences on alkaline phosphatase activity is probably moot. Indeed, it was precisely these properties of a minimum of ShlA sequences and instability resulting in loss of ShlA sequences that prompted us to select this fusion from the many available ShlA-PhoA fusions (8), because we expected that the alkaline phosphatase activity would be minimally affected by ShlA.

ACKNOWLEDGMENTS

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