JOURNAL OF BACTERIOLOGY, Oct. 1996, p. 5810–5812 Vol. 178, No. 19
0021-9193/96/$04.00+0

Identification of LytSR-Regulated Genes from *Staphylococcus aureus*

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Received 13 May 1996/Accepted 1 July 1996

In this report, the characterization of a *Staphylococcus aureus* operon containing two LytSR-regulated genes, *lrgA* and *lrgB*, is described. Sequence and mutagenesis studies of these genes suggest that *lrgA* encodes a murein hydrolase exporter similar to bacteriophage holin proteins while *lrgB* may encode a protein having murein hydrolase activity.

A region from the *Staphylococcus aureus* NCTC 8325-4 (7) chromosome was previously cloned and determined to contain two genes, designated *lytS* and *lytR*, that are involved in autolysis (2). The deduced amino acid sequences of the *lytS* and *lytR* genes share sequence similarity with sensor and response regulator proteins from the family of bacterial two-component regulatory systems, respectively. Further analysis of the DNA sequence immediately downstream from *lytR* revealed the presence of two additional open reading frames, designated *lrgA* and *lrgB* (Fig. 1A). The predicted amino acid sequence of the *lrgA* gene product (LrgA) contains 148 amino acids and has a deduced molecular mass of 16.3 kDa and a pI of 10.4. Immediately adjacent to *lrgA* is another open reading frame, termed *lrgB*, that potentially encodes a 233-amino-acid protein (LrgB) with a predicted molecular mass of 25.1 kDa and a pI of 10.6. Comparison of the deduced amino acid sequences of LrgA and LrgB against the GenBank database revealed that these proteins share amino acid sequence similarity with the hypothetical YohJ (23.6% identity and 57.5% similarity) and YohK (33.3% identity and 56.0% similarity) proteins from *Escherichia coli* and the hypothetical YwbH (30.9% identity and 67.5% similarity) and YwbG (32.6% identity and 60.3% similarity) proteins from *Bacillus subtilis*, respectively. Interestingly, the *E. coli* YohJ and YohK structural genes map near two potential *lytS* and *lytR* homologs (2), designated *yehU* and *yehT*. On the basis of sequence similarities and the conserved structural arrangement of their genes, it is possible that these regions of the *E. coli* and *S. aureus* chromosomes are both involved in cell wall metabolism. Consistent with this is the finding that the gene encoding penicillin-binding protein 7 (PBP7) is also located in this region of the *E. coli* chromosome (5).

A possible clue to the function of LrgA comes from the finding that this protein has sequence characteristics in common with the bacteriophage murein hydrolase transporter family of proteins known as holins (10). While the amino acid sequences of holins are quite divergent, they all contain structural features that are unique to these proteins. These features include small size (60 to 145 amino acids), two or more putative membrane spanning domains (often separated by a predicted beta-turn linker region), a hydrophilic N terminus, and a highly polar, charge-rich C-terminal domain (10). The LrgA protein contains four putative membrane spanning domains, two potential linker regions, and a charge-rich amino-terminal domain. On the basis of the observation that some murein hydrolases lack N-terminal signal sequences, it has been speculated that holin-like proteins that are involved in the transport of bacterial murein hydrolases might exist (9). In support of this hypothesis was the recent identification of several genes (designated *arp*) that are believed to be involved in the export of *Enterococcus hirae* murein hydrolases (3). One of these genes (*arpQ*) encodes a protein that also contains structural similarities to holin proteins (1). Although the structural similarities of the *arpQ* gene product and LrgA suggest that these proteins may have holin-like activity, the roles that they play in murein hydrolase transport have not been determined.

Transcriptional regulation of *lrgA* and *lrgB*. To determine if *lrgA* and/or *lrgB* transcription is affected by LytS and LytR, a Northern (RNA) blot analysis was employed with RNA isolated from strain 8325-4 and its *lytSR* mutant derivative, KB300 (2). The data in Fig. 1B demonstrate that *lrgA*- and *lrgB*-specific transcripts are produced in strain 8325-4 (lanes 1 and 3). An *lrgA*-specific probe hybridized to a 1.2-kb transcript in RNA isolated from 8325-4 (Fig. 1B, lane 1) that is sufficient to span the *lrgA* and *lrgB* genes (a total of 1,136 bp). Interestingly, the *lrgB*-specific probe also hybridized to a 1.2-kb transcript from 8325-4 (Fig. 1B, lane 3), suggesting that the two genes are cotranscribed. However, this probe also hybridized to a smaller, 0.8-kb transcript whose length is sufficient to span the 702-bp *lrgB* gene. Whether this transcript resulted from promoter activity near the 3′ end of *lrgA* or a specific degradation event has not yet been determined. When KB300 RNA was used, neither the 1.2-kb transcript nor the 0.8-kb transcript were detected with either the *lrgA*-specific probe (Fig. 1B, lane 2) or the *lrgB*-specific probe (Fig. 1B, lane 4). Both of these transcripts are distinct from the previously characterized 2.5-kb *lytSR* transcript (2), indicating that the effect of the *lytS* mutation in KB300 is not polar and that *lrgAB* transcription is regulated by LytS and LytR.

To identify the transcription start site for the *lrgAB* transcript, primer extension analysis was employed. In vitro extension of a synthetic oligonucleotide with RNA isolated from 8325-4 (4.5 and 10 h postinoculation) resulted in the synthesis of a 54-bp cDNA species (Fig. 2A) that localized the transcription start sites to adenine residues 18 and 19 bp upstream from the *lrgA* translation start codon (Fig. 2B). Upstream of the transcription start site is a DNA sequence (CACGCA-N_{17}-TATACT) (Fig. 2B) that matches 5 of 12 bases of the prototypical σ^A promoter of *B. subtilis* (6). Notably, more *lrgAB* tran-

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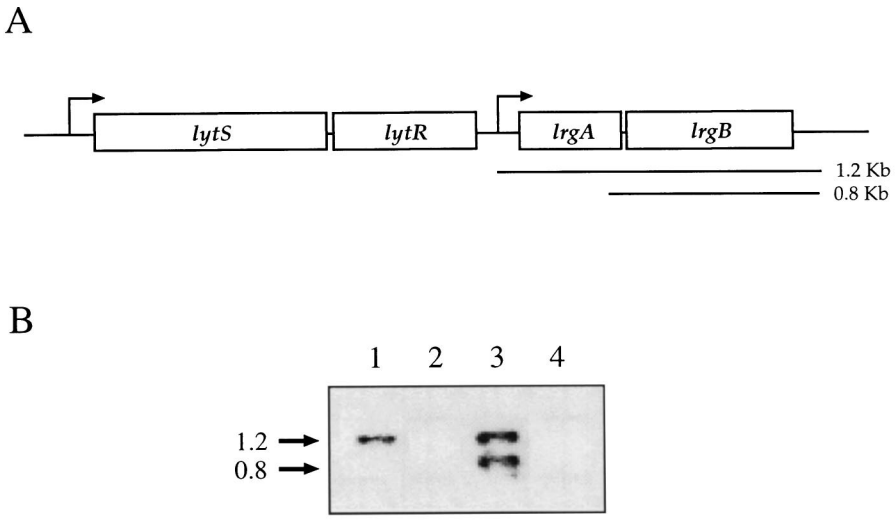

FIG. 1. (A) Organization of the *S. aureus lrgAB* region. The *lytS* and *lytR* genes encode sensor- and response regulator-like proteins, respectively (2). Approximate sizes of *lrgAB* transcripts detected by Northern blot analysis are represented by lines, with the sizes of transcripts indicated. The direction of transcription is indicated by arrows. (B) Transcription analysis of *lrgA* and *lrgB*. Northern blotting was performed with an *lrgA*-specific probe (lanes 1 and 2) and an *lrgB*-specific probe (lanes 3 and 4) against RNA isolated from 8325-4 (lanes 1 and 3) and KB300 (lanes 2 and 4), respectively. *S. aureus* RNA was isolated with RNAzol as described by Hart et al. (4). Equal amounts of RNA (20 μg) were separated in a 1.2% agarose gel (8) and transferred to nitrocellulose paper as described previously (2). The *lrgA*-specific probe (nucleotides 355 to 615; see panel A) and the *lrgB*-specific probe (nucleotides 1066 to 1384; see panel A) used in these studies were generated by PCR amplification and were radiolabeled with [α-^{32}P]ATP (8). The sizes of the transcripts, indicated on the left (in kilobases), were determined from RNA molecular weight markers (Promega).

script was detected in RNA isolated during the transition from the exponential to the stationary phase of growth (Fig. 2A, lane 1) than in RNA isolated from mid-exponential-phase cells (Fig. 2A, lane 2), indicating that *lrgAB* transcription may be temporally regulated.

Analysis of a *lrgB* mutation. Given that LytS and LytR affect autolysis in *S. aureus* and are potential transcriptional regulators of *lrgAB* expression, it was hypothesized that *lrgA* and/or *lrgB* encodes proteins that also affect autolysis. Furthermore, given the structural similarities of LrgA to holins, as described above, and the similar structural arrangement of holin and bacteriophage murein hydrolase genes (10), it seemed possible that *lrgB* might encode a murein hydrolase. Therefore, to characterize the function of this gene, an *lrgB*-specific mutation was generated. A 392-bp DNA fragment internal to the *lrgB* gene was amplified by PCR with the oligonucleotide primers 5′-GGGGGAATTCGTATGGTATTTGGTGTGG-3′ and 5′-GGGGGAATTCCGAAATTCTTAAATAATGGA-3′. The PCR product was ligated into the shuttle vector pER924 and used to disrupt the *S. aureus* 8325-4 *lrgB* gene by Campbell integration as previously described (2). Evaluation of this *lrgB* mutant, designated KB343, indicated that it had a growth rate similar to that of the wild type (1), suggesting that *lrgB* is not essential for cell growth under normal laboratory conditions. In addition, there were no differences in the rates of autolysis of KB343 and the wild type (1). However, while a zymographic analysis revealed no differences in the expression of extracellular and intracellular murein hydrolases (Fig. 3, lanes 1 to 4), a 25- to 30-kDa murein hydrolase was absent in the cell wall fraction of this strain (Fig. 3, lane 6). Furthermore, the expression of this cell wall-associated murein hydrolase is dependent on functional *lytS* and *lytR* genes, as indicated by its absence in KB300 (Fig. 3, lane 7), which has pleiotropic alterations in murein hydrolase activity, as previously reported (2). These results, along with the size of the deduced *lrgB* protein product (25.1 kDa), suggest that *lrgB* may encode this cell wall-associated murein hydrolase. However, given the hydrophobic na-

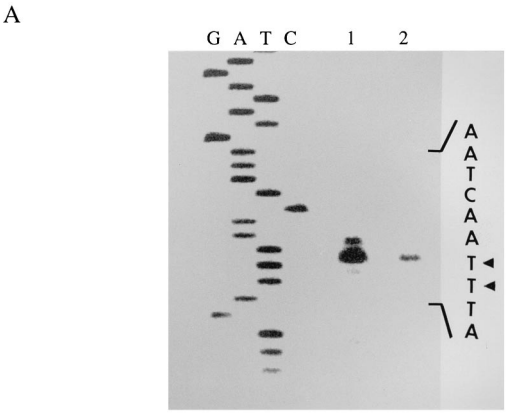

FIG. 2. (A) Primer extension analysis of the *lrgAB* transcription start site. *S. aureus* 8325-4 RNA (25 μg) was isolated from stationary-phase (lane 1) and exponential-phase (lane 2) cells and used in primer extension reactions as described previously (2). The sizes of the extended products were determined by comparison with a DNA sequencing ladder of the *lrgAB* promoter region. The primer extension and sequencing reactions were performed with the same primer. Arrowheads indicate the 5′ ends of the *lrgAB* transcripts. (B) The *lrgAB* promoter region. The nucleotide sequences of *lrgA* and *lrgB* were determined with the Sequenase DNA sequencing kit (United States Biochemical) and oligonucleotide primers specific for sequences within the *lrgA-lrgB* region. Shown are 240 nucleotides spanning the intragenic region between *lytR* and *lrgA*. The transcription start site is indicated by asterisks, and the putative Shine-Dalgarno (S.D.) and promoter elements are underlined.

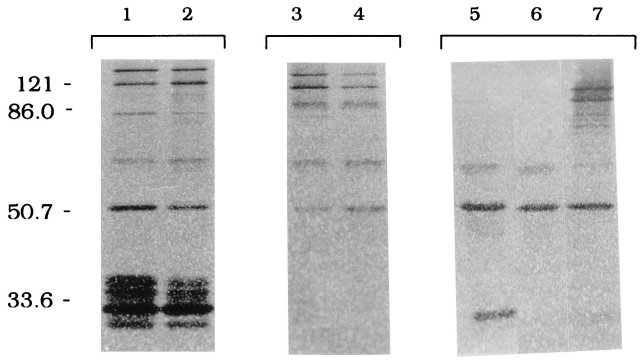

FIG. 3. Zymographic analysis of strains 8325-4, KB343, and KB300. Protein fractions (15 μg) were separated in 12.5% polyacrylamide gels containing *Micrococcus luteus* cells as described previously (2). Lanes 1 and 2, extracellular extracts of NCTC 8325-4 and KB343, respectively; lanes 3 and 4, intracellular proteins of 8325-4 and KB343, respectively; lanes 5, 6, and 7, cell wall-associated murein hydrolases of 8325-4, KB343, and KB300, respectively. Molecular mass standards are indicated in kilodaltons.

ture of LrgB (1) and the lack of sequence similarity to known murein hydrolases, the possibility that it is required for the activity or transport of this cell wall-associated murein hydrolase cannot be excluded.

The information provided in this report sheds new light on the molecular biology of murein hydrolase expression in *S. aureus*. Furthermore, the apparent conservation of these genes in *E. coli* and other bacteria (see above) suggests that these genes provide highly conserved functions. Therefore, continued study of the *lyt* regulatory locus could provide valuable insight into the regulation of murein hydrolase gene expression and cell wall metabolism in both gram-positive and gram-negative bacteria.

Nucleotide sequence accession number. The nucleotide sequences of *lrgA* and *lrgB* and their predicted protein products were submitted to GenBank (accession number U52961).

We thank Ron Yasbin, Hal Schreier, and Tammy Domanski for critical reading and insightful comments on the manuscript.

This work was supported by USDA grant 9304112 and funds from the Maryland Agriculture Experimental Station.

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