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Identification of a Gene (*arpU*) Controlling Muramidase-2 Export in *Enterococcus hirae*

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Muramidase-2 of *Enterococcus hirae* is a 74-kDa peptidoglycan hydrolase that plays a role in cell wall growth and division. To study its regulation, we isolated a mutant defective in muramidase-2 release under certain growth conditions. This mutant had cell walls which apparently lacked 74-kDa muramidase-2 but which accumulated two proteolytic fragments of 32 and 43 kDa, which exhibited muramidase-2 activity in the membrane fraction. By complementation cloning, we identified a 2.6-kb fragment of the *E. hirae* chromosome containing a gene cluster coding for proteins of 58 to 137 amino acids. One of these genes (*arpU*), which encoded a 15.9-kDa protein, was shown to complement the defect of the A9 mutant in *trans*. We propose that this gene may be involved in the regulation of muramidase-2 export.

Peptidoglycan hydrolases are enzymes responsible for a number of important biological functions such as cell wall growth, cell separation, peptidoglycan turnover, competence for genetic transformation, sporulation, and bacteriolysis induced by beta-lactam antibiotics (7, 8, 14, 21, 23, 27). Regulation of autolysin activity is believed to occur mostly at the posttranslational level. Possible regulatory mechanisms include enzyme activation by changes in substrate structure, topological restriction of enzyme distribution in the cell wall, and control at the site of export (8).

Two autolytic enzymes with muramidase activity have been described in *Enterococcus hirae*. Muramidase-1 is synthesized in a latent 130-kDa form, which is transported to specific sites in the cell wall, where it is converted to the active 87-kDa form (10). This enzyme can hydrolyze *E. hirae* cell walls but not *Micrococcus luteus* cell walls. The second enzyme, peptidoglycan hydrolase-2, or muramidase-2, is found in the culture medium during cell growth and is active on cell walls of *M. luteus* and purified peptidoglycan of *E. hirae* (5, 11). It has been postulated that muramidase-2 may operate in conjunction with muramidase-1 in peptidoglycan hydrolysis (2) and may facilitate cell separation (14, 24). The *E. hirae* muramidase-2 gene has recently been cloned and sequenced (3, 9).

In an attempt to gain insight into the mechanisms that control muramidase-2 activity in *E. hirae*, we have characterized a mutant (A9) with medium-dependent impairment of muramidase-2 export. By functional complementation of the mutant with an *E. hirae* genomic bank, we cloned a gene (*arpU*) that would appear to be involved in muramidase-2 export.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Unless stated otherwise, *E. hirae* wild-type (ATCC 9790) and mutant cells were grown at 37°C in N medium (1% Bacto Peptone [Difco Laboratories, Detroit, Mich.], 0.5% yeast extract [Difco], 1% $Na_2HPO_4 \cdot 2H_2O$). For some experiments, brain heart infusion (BHI; Difco) was used. To monitor growth, the optical density at 650 nm was measured in an LKB Ultrospec II.

Selection of mutants. For the isolation of mutants deficient in muramidase-2, wild-type *E. hirae* was mutagenized as follows. Cells in mid-log phase were washed in 1 volume of 0.1 M sodium phosphate buffer (pH 7) and suspended in 0.5 volume of the same buffer. Mutagenesis was effected by incubation with 1.6% ethyl methanesulfonate at 37°C for 60 min with agitation followed by washing of the cells twice with sodium phosphate buffer. The cells were then grown for 2 h in M17 medium (26), and 15% glycerol was added for freezing at −70°C. For mutant selection, mutagenized cells from frozen stocks were grown on N medium plates (350 CFU per plate) and muramidase-2-deficient mutants were selected by replicating the colonies onto N medium plates that had been overlaid with 2.5 ml of soft agar (0.7% agar in N medium) containing heat-killed *M. luteus* cells at an optical density of 1. Colonies that failed to develop a halo on these *M. luteus* indicator plates during overnight incubation were subcloned for further study.

Measurement of autolysis. *E. hirae* wild-type or mutant cells were grown in 20-ml cultures in N medium to 0.4 optical density unit, collected by centrifugation, washed twice with 10 ml of ice-cold 10 mM sodium phosphate buffer (pH 7.2), and resuspended in 10 ml of 0.3 M sodium phosphate buffer (pH 7.2). These suspensions were incubated at 37°C, and cell lysis was monitored turbidimetrically at 650 nm at 20-min intervals.

Detection of muramidase-2 activity. Cells were grown in 100 ml of N medium to 0.4 optical density unit, collected by centrifugation, and washed twice with ice-cold 10 mM sodium phosphate buffer (pH 7.2). The cells were resuspended in 10 ml of the same buffer and broken by ultrasonic treatment (Labsonic 2000; Braun). The total muramidase-2 activity in the resulting crude cell lysate (cell-bound muramidase-2 activity) was determined. In other experiments, protoplasts were made by resuspending the cells in 10 ml of 0.5 M sucrose–0.02 M maleic acid–0.02 M $MgCl_2$ (pH 6.5) and treating them with 4 mg of lysozyme per ml for 120 min at 37°C. The protoplasts were centrifuged at low speed, and the supernatant was preserved for determination of lysozyme-released muramidase-2 (peptidoglycan-bound enzyme). Protoplasts were then broken by ultrasonic treatment, and whole cells were removed by low-speed centrifugation. The supernatant was centrifuged at 100,000 × *g* for 60 min at 4°C to separate membranes from cytoplasm.

Proteins with muramidase-2 activity contained in these preparations were visualized on sodium dodecyl sulfate–10% polyacrylamide gels containing 0.2% (wt/vol) heat-killed *M. luteus* cells, as described by Leclerc and Asselin (13). Bands with lytic activity were observed as clear zones in the opaque gel after incubation in 25 mM Tris-Cl (pH 8)–1% Triton X-100 for 2 to 16 h at 37°C. This treatment allowed renaturation of proteins and recovery of enzymatic activity.

Gene bank construction and transformation of *E. hirae*. Genomic *E. hirae* DNA was isolated as described previously (17) and digested to completion with *Eco*RI and *Bsc*I (an isoschizomer of *Cla*I), the fragments were size fractionated on an agarose gel, and 4- to 8-kb fragments were isolated and ligated into the *Escherichia coli-E. hirae* high-copy-number shuttle vector pC3 (25), cut with *Eco*RI and *Bsc*I.

The ligation products were transformed into *E. coli* ED8739 (25), and the transformants were pooled and propagated further for the isolation of a plasmid pool from this gene bank. Plasmid DNA was isolated as described by Anderson and McKay (1). For other molecular biology methods, the protocols of Maniatis et al. (16) were followed. *E. hirae* cells were transformed with plasmid DNA by electroporation as previously described (25).

DNA sequencing and analysis. Plasmid DNA was sequenced directly with the Pharmacia T7 sequencing kit and custom-made synthetic primers. A complete set of overlapping partial sequences was derived for both DNA strands. Se-

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quence compilation and analysis were carried out with the GCG Sequence Analysis Software Package of the University of Wisconsin (4).

Plasmid constructions. Plasmid pEB79 was obtained by digestion of pEB41 with *Cla*I and *Eco*RV, filling of the sticky end with Klenow polymerase, and religation. pEB75 was generated by deleting pEB41 from the *Bst1107* site to a *Bst1107* site in the vector just beyond the cloning site.

Nucleotide sequence accession number. The DNA sequence presented here has been deposited in the EMBL database under accession number Z50854.

RESULTS

Isolation of a muramidase-2 mutant. When wild-type *E. hirae* cells are plated on solid growth media containing heat-killed cells of *M. luteus*, a clear halo develops around the colonies. This halo formation is due to the activity of muramidase-2 that is present in the *E. hirae* cell walls and released into the medium. We used this test to isolate mutants deficient in muramidase-2 function or secretion. From 3×10^3 mutagenized colonies that were tested, four strains that failed to develop a halo were isolated. In one of these mutants, A9, halo formation was medium dependent: no halos formed on N plates, but normal halos formed on BHI plates, which suggested that this was a regulatory mutant.

A9 grew at rates similar to the wild type in both media. The generation times were three times higher in N medium. A9 had a morphology indistinguishable from that of wild-type cells as judged under the light microscope and did not form long chains, as described for other lytic mutants (19, 24).

Genetic complementation of mutants. To identify the genes defective in the muramidase-2 mutant A9, the mutant was transformed by electroporation with plasmids from a wild-type *E. hirae* gene bank in pC3 (25). At a frequency of 10^{-3}, A9 yielded colonies that formed clear halos, indicating that these clones were functionally complemented by plasmids from the gene bank. With another autolysin mutant, lyt-14, whose defect was ascribed to overproduction of an enzyme repressor (24), no complemented clones could be detected. The complementing plasmids were isolated and propagated in *E. coli* for further analysis. The same plasmid, named pEB41, was obtained from several complemented A9 colonies.

Figure 1 shows the halo formation on *M. luteus* indicator plates by wild-type and A9 untransformed, transformed with the control plasmid pC3, and transformed with pEB41, respectively. Interestingly, pEB41 induced the formation of abnormally large halos in both A9 and wild-type cells (see also below). This indicated a possible overexpression of the *arpU* gene as a result of a gene dosage effect (pC3-derived plasmids are present at about 10 copies per cell in *E. hirae* [24a]).

Sequence analysis of pEB41. The sequence of the *E. hirae* DNA insert of pEB41 showed the end of an open reading frame followed by five small, complete open reading frames (Fig. 2). These frames were in all likelihood protein-encoding genes forming an operon. They are very densely spaced, and they all have a clearly identifiable ribosome-binding site properly spaced in relation to the ATG initiation codons. We have tentatively called these genes *arpQ*, *arpR*, *arpS*, *arpT*, and *arpU*, for autolysin regulatory proteins.

The translation products of the five complete *arp* genes have calculated molecular masses of 6.5, 11.5, 8.8, 11.4, and 15.9 kDa. They are polar, except for *arpQ*, which is predicted to form two transmembranous helices by Kyte and Doolittle hydrophobicity analysis (12). At the 5′ end of the DNA sequence was the end of another putative gene, and no promoter sequences could be identified. Downstream, there were no open reading frames longer than 13 amino acids. An open reading frame started at position 2035 and continued to the end of the cloned DNA.

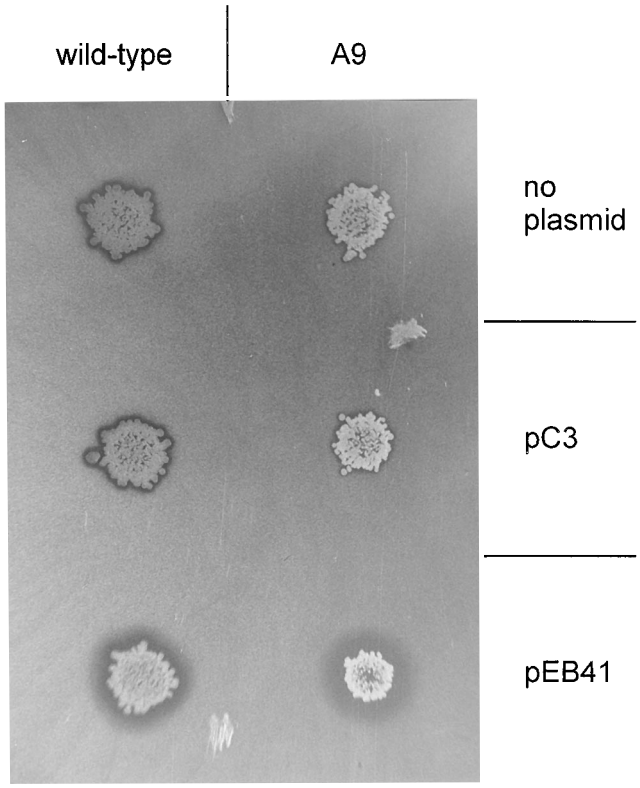

FIG. 1. Halo formation on *M. luteus* indicator plate. Samples (5 μl) of a stationary culture of the wild type and A9, both untransformed and transformed with pC3 or pEB41, were spotted onto an *M. luteus* indicator plate as shown and incubated overnight at 37°C. The different appearance of the colonies is an artifact of the tangential illumination needed to photograph the halos.

Screening the EMBL genetic database did not reveal any significant sequence similarity between these cloned genes and known sequences. In addition, we could not detect any sequence motifs that would suggest a function for the proteins.

Deletion analysis of pEB41. To identify the genes that complement A9, we constructed the deletions shown in Fig. 3. They were tested for their ability to complement A9 by transforming the mutant with the deleted plasmids and checked for halo formation. While deletion of the open reading frame at the 3′ end of the sequence (plasmid pEB79) had no effect, partial deletion of the *arpU* gene (plasmid pEB75) abolished complementation of A9. More extensive deletions also abolished the functional complementation of A9 (results not shown). This suggests that A9 has suffered mutation in the *arpU* gene and that this gene is involved in muramidase-2 release.

Characterization of the muramidase-2 mutant. The effect of incubation conditions and growth medium on muramidase-2 release in the mutant was studied. As described above, the A9 strain did not produce a halo of lysis on *M. luteus* indicator plates prepared with N medium, whereas it looked like the wild type on BHI medium indicator plates (Table 1). When A9 cells were grown on N medium indicator plates containing heat-killed *E. hirae* cells, the substrate for muramidase-1, they produced a halo of lysis like the wild type, indicating normal levels of muramidase-1. The halos produced by A9(pEB41) were bigger than those produced by the wild type under all conditions tested, suggesting that overexpression of the *arpU* gene

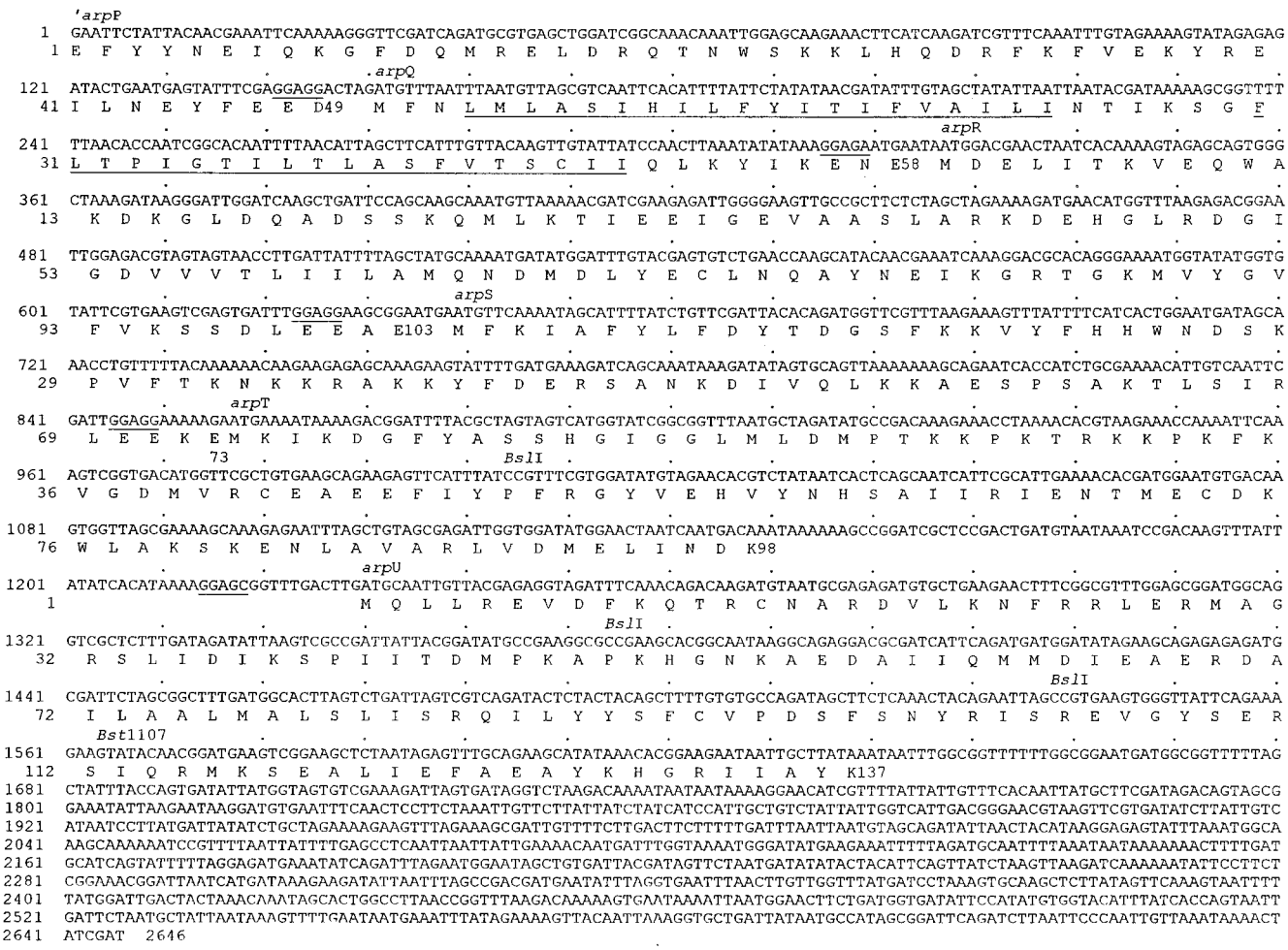

FIG. 2. Sequence of the *E. hirae* DNA insert of pEB41. The amino acid translations of the *arp* genes are given below the DNA sequence. The names of the genes and important restriction sites are indicated above the DNA sequence. Ribosome-binding sites are underlined, as are the two putative transmembranous helices of *arpQ*.

product increased the release of both muramidase-1 and muramidase-2. Muramidase-2, in conjunction with muramidase-1, is thought to be involved in autolysis of *E. hirae*. Measurements of the autolysis rates of the wild type and the mutant are shown in Fig. 4. Autolysis of strain A9 was considerably more rapid than that of the wild type or A9(pEB41). Wild-type cells harboring the pC3 cloning vector exhibited slightly faster autolysis than did untransformed cells, indicating that the presence of a plasmid per se can influence the autolysis kinetics. It has been reported that alterations in autolytic enzyme activity can influence the susceptibility to penicillin (6). We found that the MIC of penicillin for A9 was 6.25 μg/ml and was identical to that for the wild type, in both N and BHI media.

The method described by Leclerc and Asselin (13) allows detection of peptidoglycan hydrolases in sodium dodecyl sulfate-polyacrylamide gels containing 0.2% heat-killed *M. luteus* cells. Following electrophoretic separation of the proteins, they are renatured, and the ensuing muramidase-2 activity can be detected by the formation of lytic bands in the gel. This method was used to study the production of muramidase-2 by

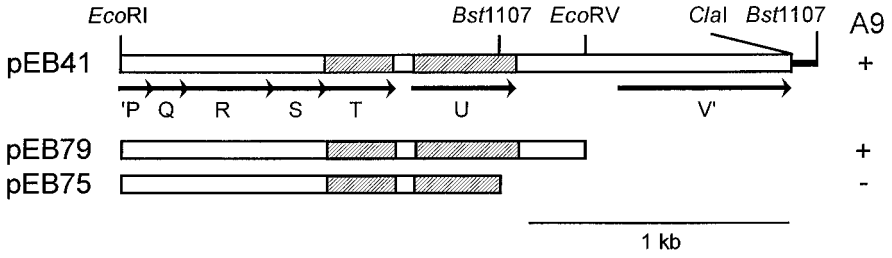

FIG. 3. Maps of the *E. hirae* DNA insert of pEB41 and its deletion derivatives. The open and hatched boxes indicate *E. hirae* DNA, and the thick black line indicates a plasmid DNA. Important restriction sites are indicated. *Eco*RI and *Cla*I are the sites used for cloning in pC3. The arrows denote open reading frames. On the right, complementation of the A9 mutant by the respective plasmids is indicated.

TABLE 1. Halo formation by different *E. hirae* strains on BHI or N medium indicator plates with heat-killed *M. luteus* or *E. hirae* cells

Strain	Halo formation on[a]:			
	BHI-*E. hirae*	BHI-*M. luteus*	N-*E. hirae*	N-*M. luteus*
Wild type	+	+	+	+
WT(pC3)	+	+	+	+
A9	+	+	+	−
A9(pEB41)	+++	++	+++	++

[a] BHI or N medium indicator plates containing either *M. luteus* or *E. hirae* heat-killed cells.

A9 and A9(pEB41) in comparison with the wild type. Figure 5 shows the protein patterns (Fig. 5A) and the pattern of lytic bands (Fig. 5B) of whole lysates of the wild type, A9, and A9(pEB41), respectively. It can clearly be seen that strain A9 produced three lytic bands, of 74, 43, and 32 kDa, whereas the wild type and A9(pEB41) produced only the 74-kDa lytic band. This lytic component was strongly reduced in A9. In agreement with this finding, a 74-kDa band was apparently lacking on the Coomassie blue-stained gel. When A9 was grown in BHI medium instead of N medium, it behaved like the wild type: only the 74-kDa lytic band was detected in the zymogram and the 74-kDa protein could be detected on the Coomassie blue-stained gel (results not shown).

Cellular location of muramidase-2. Because we suspected that the failure of A9 to develop a halo on *M. luteus* indicator plates might also be due to defects in enzyme secretion, we investigated the localization of muramidase-2 in different cellular compartments. Cytoplasm, membrane, and cell wall protein extracts were prepared from the wild type, the wild type containing pC3, A9, and A9(pEB41) and analyzed zymographically by the method used for the data in Fig. 5. As shown in Fig. 6, muramidase-2 activity was not detectable in the cytoplasm of any of the strains but was present in membranes of all strains. This finding is in agreement with results of studies conducted by others reporting that over 95% of muramidase activity is localized in cell envelopes (22).

Clearly, most of the muramidase-2 activity of A9 was localized in the membrane fraction, where the enzyme was degraded to 43- and 32-kDa components. The cell walls of A9 were found to contain significantly less enzyme than did those of the wild type. No difference in muramidase-2 production was found between wild type and wild type transformed with pC3 in this test, thus excluding an influence of the cloning vector on muramidase-2. In the experiment described in Fig. 6, the amount of the 74-kDa form produced by A9 was apparently larger than that found in the experiment in Fig. 5. This finding suggested that the ratio of the 74-kDa form to the smaller forms of muramidase-2 could be influenced by the experimental conditions. It is interesting that the experiment which showed the apparent higher accumulation of the active 74-kDa enzyme in membranes of A9 with respect to those of the wild type also revealed the lack of muramidase-2 activity in the cell wall fraction of A9. If this mutant was not impaired in the enzyme transport process, such a higher content of enzyme at the membrane level should have allowed the recovery of the wall-bound enzyme in amounts at least similar to those in the wild type.

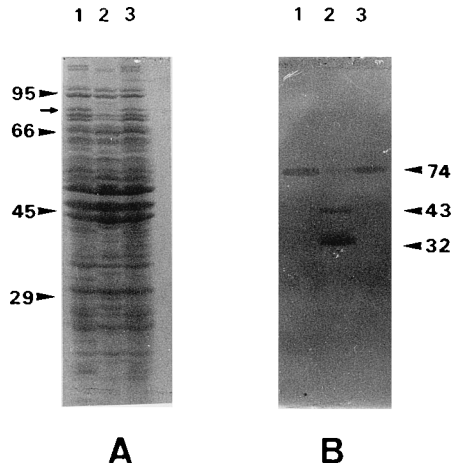

FIG. 5. Protein and lytic enzyme profiles of A9 mutant. Total-cell lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis for staining with Coomassie blue (A) and zymography with *M. luteus* as the substrate (B), respectively. Lanes: 1, wild type; 2, A9; 3, A9(pEB41). Details of the procedure are given in Materials and Methods. The arrowheads in panel A indicate the migration of molecular mass markers in kilodaltons and the arrow identifies the position of the 74-kDa muramidase-2 band. The arrowheads in panel B identify lytic bands and their molecular masses in kDa.

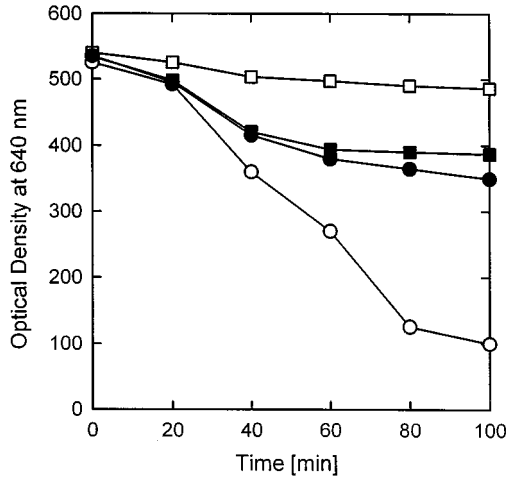

FIG. 4. Autolysis of *E. hirae* strains. Cells cultivated in N medium were washed and incubated at 37°C in 0.3 M phosphate buffer (pH 7.2), and the turbidity was monitored at 650 nm. Details of the procedure are outlined in Materials and Methods. Symbols: □, wild type; ■, wild type containing pC3; ○, A9; ●, A9(pEB41).

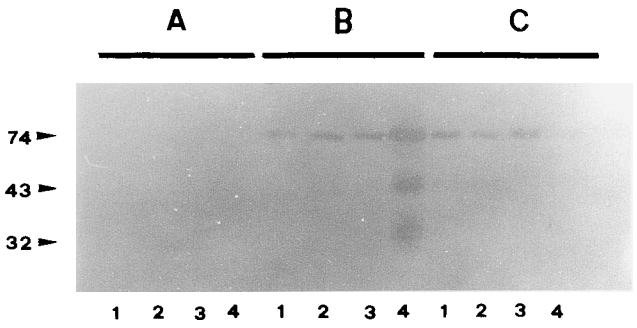

FIG. 6. Zymogram of different cellular fractions. Cells were fractionated into cytoplasm (A), membranes (B), and cell walls (C) as described in Materials and Methods, and a zymogram was produced as in Fig. 5. Lanes: 1, wild type containing pC3; 2, wild type; 3, A9(pEB41); 4, A9.

DISCUSSION

In this paper, we describe a mutant of *E. hirae*, A9, whose medium-dependent inability to form lytic halos around colonies grown on *M. luteus* indicator plates was associated with increased autolysis in phosphate buffer and with degradation of muramidase-2 into smaller fragments (43 and 32 kDa) which maintained the enzymatic activity, as well as with a lack of cell wall-bound enzyme. These results excluded the possibility that the A9 phenotype was due to a reduction in muramidase-2 activity and suggested that it was more likely to be due to a defect in the enzyme transport.

The properties of mutant A9 appeared to be very different from those of other lytic mutants of *E. hirae* (19, 24) or other gram-positive bacteria (15, 18), for which the inability to form lytic halos on *M. luteus* indicator plates has been found to be associated with a reduced autolysis rate and has been explained by a defect in enzyme synthesis or activity or by an increased production of enzyme inhibitors. Our findings that A9 did not produce lytic halos and autolysed more rapidly than the wild type were not conflicting: autolysis in phosphate buffer is triggered by cell-bound enzymes, whereas lytic halos around colonies are formed by the released enzymes. Apparently, A9 accumulated more enzyme (in particular the 43- and 32-kDa forms) than the wild type did in the cells, and this might account for the higher rate of autolysis. Defects in the muramidase-2 export process may be directly ascribed to alterations in signal (leader) peptidase or in chaperon proteins which assist in the assembly and/or export of secreted proteins (20); accumulated enzyme molecules could then be degraded by endogenous proteases. Alternatively, the abnormal activation of a protease active on muramidase-2 could increase the rate of protein degradation into forms which are no longer suitable for transport.

Complementation analysis with a genomic library of *E. hirae* allowed us to isolate a 2.6-kb fragment containing a gene (*arpU*) whose mutation was probably responsible for the A9 phenotype. This gene may be directly or indirectly involved in export of muramidase-2. It may encode a leader peptidase or a chaperon protein (direct involvement) or be involved in regulation of a protease responsible for posttranslational control of protein synthesis (indirect involvement).

Apparently, complementation by *arpU* was associated with overexpression of the wild-type product, since both A9(pEB41) and wild type harboring pEB41 produced bigger halos than did the wild type. This may suggest that the A9 defect in halo formation was nonspecifically offset by the amplified gene product. However, complementation by *arpU* also caused the disappearance of the 43- and 32-kDa forms, and this strongly supports the hypothesis that the A9 phenotype was specifically suppressed by the wild-type *arpU* gene.

The sequence of the pEB41 insert containing the *arpU* gene indicated the presence of a cluster of genes whose organization resembles that of an operon. The well-established observation that bacterial operons unite genes for associated functions suggests the possibility that the other *arp* genes are also involved in regulation of muramidase-2 export or in a more general mechanism responsible for protein processing or export.

Why cells undergo autolysis, in which muramidase-2 is involved, is unknown. Conceivably, it is merely an undesirable side effect of the enzymes responsible for cell wall synthesis. In any case, muramidase and other cell wall lytic enzymes must be carefully controlled by the cell. The export defect and the degradation of muramidase-2 in mutant A9 cells may represent a mutation affecting a physiological mechanism that controls the muramidase-2 activity reaching the cell wall, thereby avoiding dangerous consequences for bacterial integrity.

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