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Secretion of Ipa Proteins by *Shigella flexneri*: Inducer Molecules and Kinetics of Activation

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The type III Mxi-Spa secretion machinery of *Shigella flexneri* is responsible for secretion of Ipa proteins, which are involved in the entry of bacteria into epithelial cells. Ipa proteins accumulate within bacteria growing in laboratory media, and their secretion is activated upon contact of bacteria with eukaryotic cells. In this study, we have identified a group of chemical compounds, including Congo red, Evans blue, and direct orange, which are able to induce secretion of Ipa proteins by bacteria suspended in phosphate-buffered saline. Parameters of kinetics of activation of Ipa secretion by Congo red were determined by measuring by enzyme-linked immunosorbent assay the amount of IpaC secreted and by investigating the increase in susceptibility of Ipa proteins to proteinase K degradation. Ipa secretion occurred at 37°C, was obtained with 5 to 10 μM Congo red, and was complete within 30 min. In addition, activation of Ipa secretion by Congo red was observed with bacteria harvested throughout the exponential phase of growth but not with bacteria in the stationary phase. The interactions of Congo red and Congo red-related compounds with the Mxi-Spa secretion apparatus might be specific hydrophobic interactions similar to those involved in binding of Congo red to amyloid proteins.

Protein secretion in gram-negative bacteria occurs by three pathways which are characterized by two mechanisms (21). The mechanisms of secretion are designated *sec* dependent or *sec* independent, depending on the presence or the absence of a signal sequence at the N-terminal end of the proteins destined for secretion. The type I secretion pathway, exemplified by secretion of the *Escherichia coli* hemolysin, is *sec* independent and involves three accessory proteins (22). The type II, or general secretory, pathway is *sec* dependent and is divided into several branches, one of which is used for secretion of the pullulanase of *Klebsiella oxytoca* and involves 13 accessory proteins (21). The type III secretion pathway is *sec* independent and has been identified in a number of human and animal pathogens, such as *Shigella*, *Yersinia*, *Salmonella*, and enteropathogenic *E. coli*, and in plant pathogens, such as *Xanthomonas*, *Pseudomonas*, and *Erwinia* (27). This pathway is required for secretion of pathogenicity factors and involves about 20 accessory proteins, among which 7 are conserved in all the systems (28). A key feature of type III secretion machineries is that their activity, rather than their synthesis or the synthesis of proteins to be secreted, is modulated in response to interactions between bacterial pathogens and their hosts. Contact of *Shigella* and *Salmonella* with epithelial cells triggers secretion of presynthesized bacterial proteins which are involved in inducing uptake of these bacteria by epithelial cells (11, 29, 30). Likewise, contact of *Yersinia* with host cells induces secretion of a negative regulator, which derepresses expression of anti-host proteins (19). However, little is known about the mechanism of activation of type III secretion machineries and about the nature of the host signals which induce secretion.

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Shigella spp. cause bacillary dysentery in humans by invading colonic epithelial cells. Invasion of cultured cells by *Shigella flexneri* involves two integrated steps, entry and intercellular dissemination. Genes involved in both steps are carried by a 200-kb virulence plasmid (23, 25). A 31-kb fragment of this plasmid, which is necessary and apparently sufficient for entry (8, 24), contains 32 genes clustered in two divergently transcribed regions that encode secreted proteins (the IpaA through IpaD proteins) and a type III secretion machinery (the Mxi-Spa apparatus) (see reference 17 for a review). Although *mxi* and *spa* genes, encoding the secretion machinery, and *ipa* genes are expressed by bacteria growing in laboratory media, only a small proportion of Ipa proteins is actually secreted by the wild-type strain under these growth conditions. In contrast, secretion of IpaB and IpaC, the effectors of *Shigella* entry into epithelial cells (10), is induced upon contact of bacteria with epithelial cells (11, 29).

Inactivation of either *ipaB* or *ipaD* leads to a phenotype of constitutive secretion, i.e., the other Ipa proteins are secreted efficiently by *ipaB* and *ipaD* mutants growing in laboratory media (11). This indicates that IpaB and IpaD are both required to prevent secretion in the absence of inducing signals and that, in the wild-type strain, external signals are not required for assembly of the secretion machinery. We have shown previously that growth of *Shigella* in liquid media containing Congo red, a sulfonated azo dye, leads to an enhanced secretion of Ipa proteins (16). This observation led us to investigate whether Congo red could activate secretion of Ipa proteins in the absence of bacterial growth. By measuring the amount of IpaC which was secreted after exposure of bacteria to Congo red, we have determined some parameters of the kinetics of activation of secretion, such as dependency on the dose of inducer, the time and temperature of incubation in the presence of inducer, and the stage of bacterial growth. We have also identified Congo red-related dyes which induce secretion of Ipa proteins. These compounds possess a central hydrophobic moiety, as well as charged groups at their extremities.

MATERIALS AND METHODS

Bacterial strains and growth media. *S. flexneri* M90T is an invasive isolate which belongs to serotype 5 (23). Strains SF623 and SF403 are derivatives of M90T and carry *ipaA-lacZ* and *mxiD-lacZ* transcriptional fusions, respectively (1, 12). Bacteria were grown in tryptic soy (TCS) broth (Diagnostics Pasteur, Marnes la Coquette, France). Ampicillin was used at 100 μg/ml.

Activation of Ipa secretion. Precultures of bacteria grown at 37°C in TCS broth were diluted 100 times in TCS broth and incubated at 37°C under aerated conditions until the culture reached an optical density of 1 at 600 nm. The culture was centrifuged, and the pellet was resuspended in phosphate-buffered saline (PBS) (pH 7.3) such that the concentration of bacteria was 3×10^8 CFU/ml. The potential inducer was added, and the reaction mixture was incubated at 37°C for 30 min. Samples were then centrifuged at 14,000 × *g* for 15 min at 20°C, and the supernatant fractions were analyzed by enzyme-linked immunosorbent assay (ELISA) or immunoblotting. The following compounds were used at concentrations of 20 μM: Congo red (Serva, Heidelberg, Germany), acid yellow, direct orange, Evans blue, naphthol blue black, 4-amino-1-naphthalene sulfonic acid (Aldrich, Milwaukee, Wis.), adenine, thymine, tyrosine, and tryptophan (Sigma, St. Louis, Mo.).

ELISA for quantitation of IpaC. The amount of IpaC present in the supernatant fraction following an induction assay or in the lysate of sonicated bacteria was determined by ELISA with a two-antibody sandwich assay (7). Briefly, 96-well polystyrene plates were coated with the J22 anti-IpaC monoclonal antibody (20), washed, filled with serial dilutions of the supernatant fraction or sonicated whole-cell extract, and incubated for 1 h at 37°C. Plates were then washed and incubated with an anti-IpaC polyclonal antibody (20) for 1 h at 37°C. A horseradish peroxidase-conjugated anti-rabbit immunoglobulin G was used to detect binding of polyclonal antibodies. The reaction was then developed for 15 min at 20°C with *o*-phenylenediamine dihydrochloride (Sigma) as a substrate, and the optical density was read at 490 nm. The amount of IpaC was expressed in optical density units, as calculated from the plot of the optical density at 490 nm versus the dilution factors of the supernatant fraction or of the sonicated lysate.

SDS-PAGE and immunoblotting. Protein samples were denatured in Laemmli sample buffer, subjected to sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis (SDS-PAGE), and transferred onto a nitrocellulose membrane (2). Immunoblot analyses were carried out with anti-IpaC (J22) and anti-IpaB (H16) monoclonal antibodies (4, 20) and anti-IpaD (12) and anti-adenylate kinase polyclonal antibodies. Goat anti-mouse or anti-rabbit immunoglobulins conjugated to horseradish peroxidase were used as secondary antibodies and visualized by enhanced chemiluminescence.

Proteinase K digestion. After incubation of bacteria in the presence of Congo red, a fraction of the reaction mixture was incubated with proteinase K (10 μg/ml) for 10 min at 20°C. Proteinase K was then inactivated by addition of phenylmethylsulfonyl fluoride (2 mM), and samples were analyzed by SDS-PAGE and immunoblotting.

β-Galactosidase assay. β-Galactosidase activity was assayed with the substrate *o*-nitrophenol-β-D-galactoside and was expressed in Miller units (13).

RESULTS

Activation of Ipa secretion by Congo red and related compounds. The observation that the presence of Congo red in the growth medium of *Shigella* enhances secretion of Ipa proteins (16) led us to investigate whether secretion of Ipa proteins could be activated by Congo red in the absence of bacterial growth. Bacteria in the exponential phase of growth were harvested by centrifugation, resuspended in PBS, and incubated in the presence of Congo red (10 μM) for 30 min at 37°C. Samples were centrifuged, and the presence of IpaB, IpaC, and IpaD in the supernatant fraction was analyzed by Western blotting (Fig. 1). No Ipa proteins were detectable in the supernatant of bacteria that had been incubated in PBS alone, whereas they were present in the supernatant of bacteria that had been incubated in the presence of Congo red. Adenylate kinase, a cytoplasmic enzyme, was not detected in the supernatant fraction, which confirmed that release of Ipa proteins was not due to bacterial lysis. These results indicated that Congo red was able to induce secretion of Ipa proteins stored within the cytoplasmic compartment and that no cofactors that could have been present in the TCS broth or secreted by bacteria in the growth medium were required for secretion.

In an attempt to define which part of the Congo red molecule was important for activation of secretion, we have tested other dyes which share structural features with Congo red,

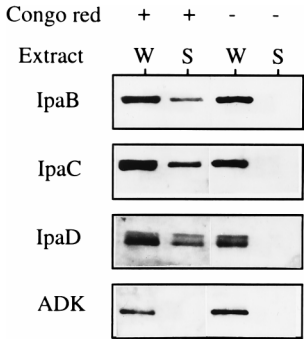

FIG. 1. Activation of Ipa secretion by Congo red. Bacteria were suspended in PBS at a concentration of 3×10^8 CFU/ml and incubated at 37°C in the presence or absence of 10 μM Congo red. After 30 min of incubation, aliquots of whole samples (W) were withdrawn and kept at 20°C while the rest of the samples was centrifuged at 20°C to prepare the supernatant fractions (S). Identical volumes of whole-sample and supernatant extracts were subjected to SDS-PAGE and immunoblotted with antibodies directed against IpaB, IpaC, IpaD, and adenylate kinase (ADK).

such as a central hydrophobic core or charged groups (Fig. 2). Both Evans blue and direct orange activated secretion of IpaB, IpaC, and IpaD, whereas acid yellow-42, naphthol blue black, and 1-naphthylamine-4-sulfonic acid did not (Fig. 3). Likewise, physiological aromatic compounds, such as nucleotides (adenine and thymine) and amino acids (tyrosine and tryptophan), were not able to induce Ipa secretion (data not shown).

Kinetics of activation of Ipa secretion by Congo red. To investigate the kinetics of activation of secretion, a quantitative ELISA was set up to determine the amount of IpaC that was secreted in response to contact of bacteria with Congo red.

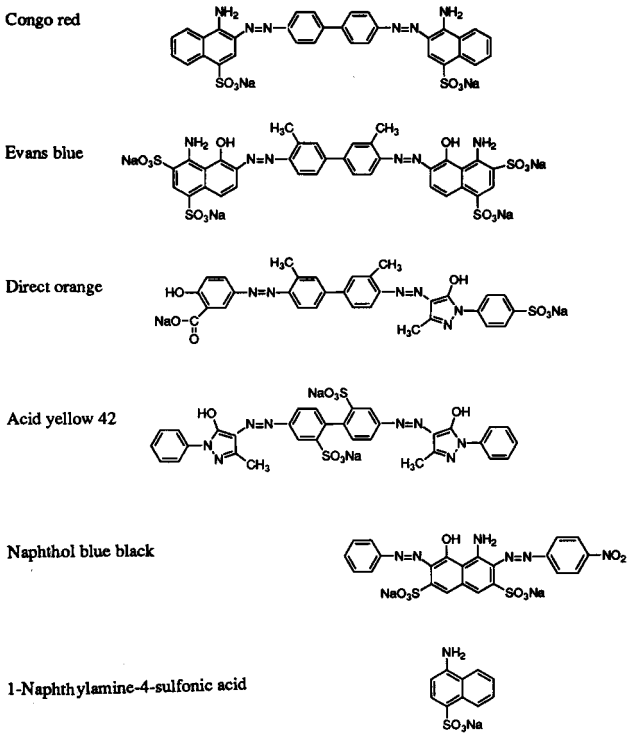

FIG. 2. Structure of Congo red and related compounds tested for activation of Ipa secretion.

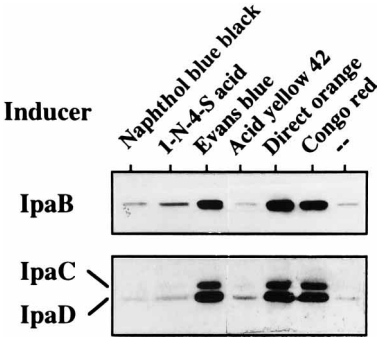

FIG. 3. Activation of Ipa secretion by Congo red and related compounds. Bacteria were suspended in PBS and incubated at 37°C for 30 min in the presence of various potential inducers at 20 μM. Samples were centrifuged, and supernatants were analyzed by SDS-PAGE and Western blotting with anti-IpaB, anti-IpaC, and anti-IpaD antibodies. 1-N-4-S acid, 1-naphthylamine-4-sulfonic acid.

First, induction of secretion was carried out by using various concentrations of Congo red. Results of a representative experiment are shown in Fig. 4A. Secretion of IpaC was obtained in the presence of 10 μM Congo red, and no distinct increase in the amount of secreted IpaC was observed as the concentration of Congo red was increased up to 1 mM. Secretion of IpaC and IpaB in response to various concentrations of Congo red, Evans blue, and direct orange was also analyzed by Western blotting of the supernatant fraction (Fig. 4B). Maximal secretion of Ipa proteins was obtained by using 20 μM Evans blue or direct orange and with less than 10 μM Congo red.

We have tested the activation of IpaC secretion at 4 and 23°C, the temperatures which are used to maintain bacteria prior to invasion assays. No secretion of IpaC was induced by Congo red at these temperatures (data not shown), which suggested that the Mxi-Spa secretion machinery or the Congo red activation pathway is inactive at low temperatures.

Secretion of IpaC was also analyzed as a function of the time of incubation in the presence of 10 μM Congo red. Maximal recovery of IpaC in the supernatant was obtained after 30 min of incubation (Fig. 5). As estimated by the ELISA, the amount of IpaC present in the supernatant fraction following 30 min of incubation in the presence of Congo red corresponded to approximately 40% of the pool of IpaC present in bacteria. This estimation was confirmed by Western blot analysis of the supernatant fraction (Fig. 6). To determine whether the IpaC that was present in the bacterial pellet after centrifugation of the samples still remained in the cytoplasmic compartment, samples were treated with proteinase K and analyzed by SDS-PAGE and immunoblotting. In this assay, cytoplasmic proteins are protected from degradation by proteinase K, whereas membrane-associated proteins and secreted proteins are degraded. Following incubation of bacteria in the presence of Congo red, proteinase K was added, and the reaction mixtures were incubated for 10 min at 20°C, a temperature at which the secretion machinery is not active (see above). After 30 min of incubation in the presence of Congo red, the total pool of IpaC was susceptible to degradation by proteinase K (Fig. 6). This indicated that the localization of most of IpaC had been modified upon incubation in the presence of Congo red, even though only 40% of IpaC was recovered in the supernatant fraction. Likewise, the total pool of IpaB and IpaD was susceptible to proteinase K degradation, although, as in the case of IpaC, only a fraction of these antigens was present in the supernatant (Fig. 6). In response to induction by Congo red,

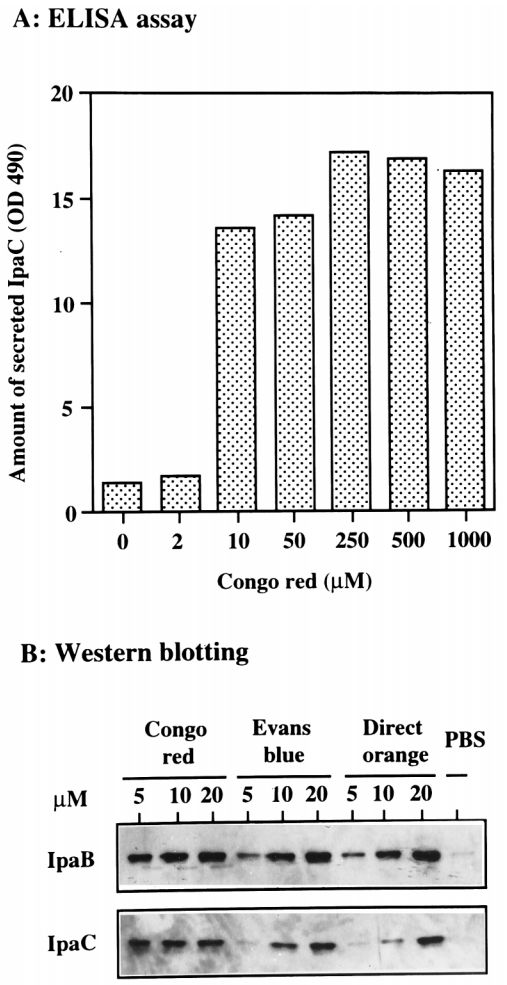

FIG. 4. Activation of Ipa secretion at various concentrations of inducers. Bacteria were suspended in PBS and incubated in the presence of various concentrations of Congo red, Evans blue, or direct orange. After 30 min of incubation at 37°C, samples were centrifuged to prepare the supernatant fraction. The amount of IpaC present in the supernatant fraction was determined by ELISA and is expressed in units of absorbance at 490 nm (A). OD 490, optical density at 490 nm. Supernatant fractions were also analyzed by SDS-PAGE and Western blotting with anti-IpaB and anti-IpaC antibodies (B).

Ipa proteins might become exposed at the bacterial surface and only a certain proportion of the proteins would actually be released into the external milieu. Alternatively, the incomplete recovery of Ipa proteins in the supernatant might be due to aggregation of secreted proteins and sedimentation of these aggregates during the centrifugation used to prepare the supernatant fraction. The latter hypothesis is supported by the observation that prolonged incubation, i.e., for 1 h and longer, of bacteria in the presence of Congo red led to a decrease in the amount of IpaC present in the supernatant fraction (Fig. 5 and data not shown).

Since the measure of the amount of Ipa proteins present in the supernatant fraction might underestimate the activity of the secretion machinery, we investigated the kinetics of activation of Ipa secretion by using the proteinase K sensitivity test. Samples containing bacteria which had been incubated in the presence of Congo red for 15, 30, or 60 min at 37°C were supplemented by addition of proteinase K, incubated at 20°C for 10 min, and analyzed by SDS-PAGE and immunoblotting

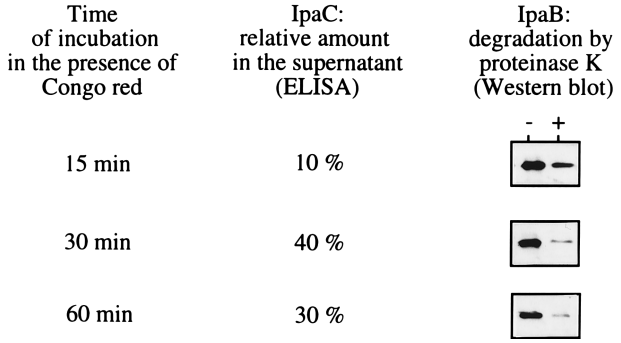

FIG. 5. Kinetics of activation of Ipa secretion by Congo red. Bacteria were suspended in PBS and incubated in the presence of 10 μM Congo red. After various times of incubation at 37°C, samples were either incubated in the presence of proteinase K for 10 min at 20°C and analyzed by Western blotting with an anti-IpaB antibody or were centrifuged to determine by ELISA the amount of IpaC present in the supernatant fraction. The amount of IpaC that was present in the supernatant is expressed relative to the amount of IpaC present in sonicated extracts of bacteria (100%).

with anti-IpaB antibodies. As shown in Fig. 5 (right panel), IpaB was susceptible to degradation by proteinase K after 15 min of incubation in the presence of Congo red.

In conclusion, for bacteria harvested during the exponential phase of growth, secretion of Ipa proteins in the presence of Congo red was (i) achieved by using micromolar concentrations of Congo red, (ii) temperature dependent, (iii) rapid, with a significant proportion of Ipa proteins secreted or exposed at the bacterial surface within 15 min, and (iv) complete in 30 min.

Activation of Ipa secretion with respect to the bacterial growth phase. To investigate the relationship between the response of the secretion machinery to external inducers and the stage of bacterial growth, bacteria were harvested at different stages of growth and incubated in the presence of Congo red. Secretion of IpaC was measured by ELISA. Although the same number of bacteria was used in each case, approximately six times more IpaC was secreted by bacteria at the end of the exponential phase of growth than by bacteria in the early exponential phase of growth (Fig. 7). In addition, bacteria in the late stationary phase, i.e., after 24 h of incubation at 37°C,

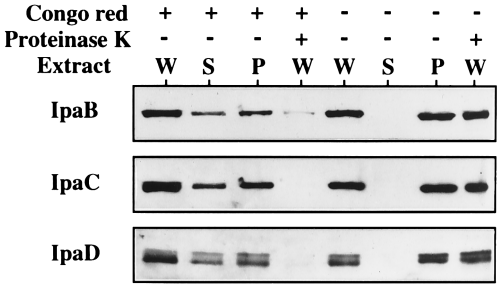

FIG. 6. Proteinase K susceptibility of Ipa proteins following incubation of bacteria in the presence of Congo red. Bacteria suspended in PBS were incubated in the presence or absence of Congo red for 30 min at 37°C. Aliquots of the samples were withdrawn and either kept at 20°C (W, whole sample) or centrifuged at 20°C to prepare the supernatant fraction (S) and the bacterial pellet (P), which was then resuspended in the initial volume of PBS. The rest of the samples were supplemented by addition of proteinase K (10 μg/ml), and, after 10 min of incubation at 20°C, proteinase K was inhibited by addition of phenylmethylsulfonyl fluoride. Identical volumes of whole samples which had been left untreated or treated with proteinase K, supernatants, and resuspended pellets were analyzed by SDS-PAGE and Western blotting with anti-IpaB, anti-IpaC, and anti-IpaD antibodies.

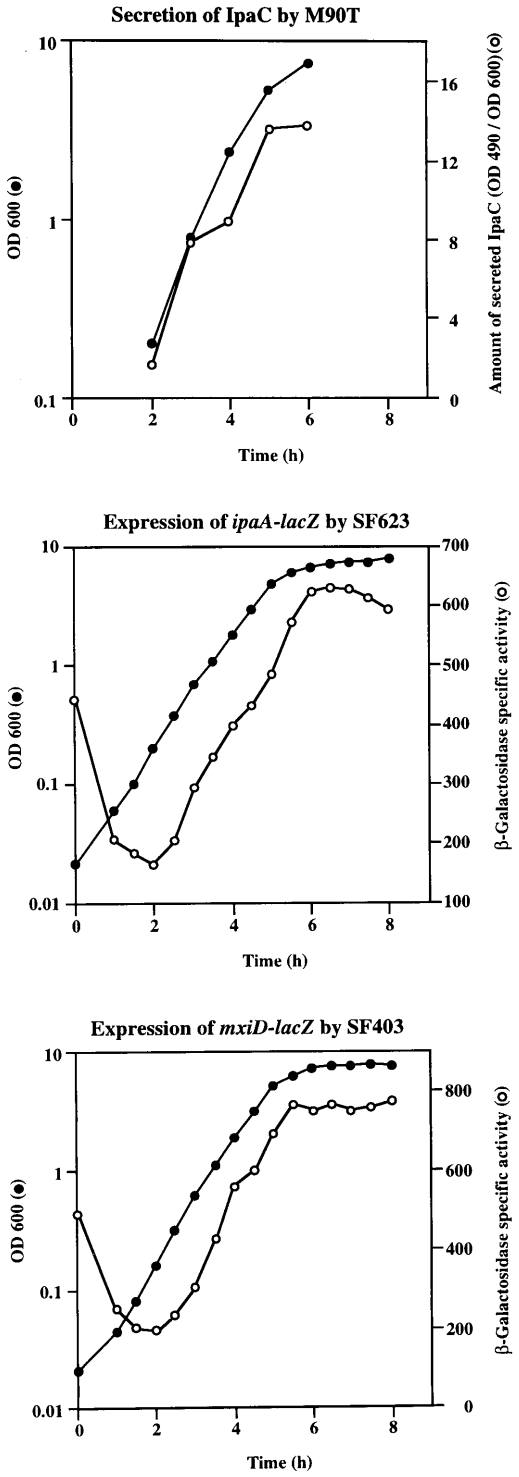

FIG. 7. Induction of IpaC secretion by Congo red and expression of *ipaA-lacZ* and *mxiD-lacZ* at various stages of growth. At various times after dilution of strains M90T (wild type), SF623 (*ipaA-lacZ*), or SF403 (*mxiD-lacZ*) in TCS broth and incubation at 37°C, aliquots of the cultures were withdrawn to measure the optical density (OD) of cultures at 600 nm and to test the ability of these bacteria to be activated by Congo red for secretion of IpaC (M90T) or to assay β-galactosidase activity (SF623 and SF403). The amount of IpaC secreted was determined by ELISA and is expressed in units of optical density at 490 nm per unit of optical density at 600 nm. β-Galactosidase activity is expressed in Miller units.

were not activated by Congo red to secrete Ipa proteins, as determined by measuring the release of IpaC by ELISA or the susceptibility of IpaB to proteinase K degradation (data not shown). The inability of bacteria in stationary phase to secrete Ipa proteins has also been observed when serum was used as the inducer (14).

To determine whether the difference in IpaC secretion observed between bacteria taken early and late in the exponential phase of growth was the result of a differential production of secretory (Ipa) or secretion (Mxi and Spa) proteins, we used *lacZ* transcriptional fusions to investigate the expression of the *ipa* and *mxi* operons at various stages of growth. Strains SF623 and SF403 carrying *ipaA-lacZ* and *mxiD-lacZ* transcriptional fusions, respectively, were grown in TCS broth at 37°C, and at various time points aliquots were withdrawn to measure the optical density at 600 nm and to assay β-galactosidase activity (Fig. 7). For both strains, bacteria at the end of the exponential phase of growth (6 h of incubation) contained four times more β-galactosidase than bacteria taken early in the exponential phase of growth (2 h of incubation). Therefore, the larger amount of IpaC secreted by bacteria at the end of the exponential phase was probably due to the accumulation of Ipa, Mxi, and, presumably, Spa proteins rather than to a modification of the ability of the secretion machinery to be activated by Congo red.

DISCUSSION

The signals necessary for activation of type III secretion machineries are provided by contact between bacteria and host cells (11, 19, 29, 30). The induced secretion of presynthesized proteins might allow bacteria to deliver a high local concentration of effector proteins onto the surfaces of target cells. In addition to contact with target cells, incubation of bacteria in the presence of serum stimulates secretion of Ipa proteins by *Shigella* (11, 14) and of InvJ by *Salmonella* (30). This suggests that different molecules or possibly the same molecule in both soluble and membrane-bound forms can activate the type III secretion machineries of *Shigella* and *Salmonella*. We have shown previously that growth of *Shigella* in a culture medium containing Congo red enhances secretion of Ipa proteins (16). To get further insight into the kinetics and possibly the mechanism of activation of the secretion machinery of *Shigella*, we have investigated the ability of this artificial but well-defined molecule to induce Ipa secretion. Bacteria in the exponential phase of growth were harvested and resuspended in PBS so that secretion studies could be performed on a defined pool of presynthesized Ipa proteins.

Following incubation of bacteria in the presence of Congo red, the activity of the secretion machinery was determined by measuring the amount of Ipa proteins in the supernatant, and we found that (i) Ipa secretion is activated at 37°C but not at 4 or 20°C, (ii) activation of secretion is obtained at micromolar concentrations of Congo red, (iii) the entire pool of cytoplasmic Ipa proteins becomes accessible to proteinase K within 30 min, and (iv) the secretion machinery can be activated throughout the exponential phase of growth but not during the stationary phase, i.e., after 24 h of incubation at 37°C. One possible explanation for the poor secretion by bacteria in stationary phase is that some components of the secretion machinery might be missing in these bacteria, due to their intrinsic instability. The lack of secretion might also be due to the inability of these bacteria to provide energy for secretion. Alternatively, Ipa proteins stored in the cytoplasmic compartment might be in a state that is no longer competent for secretion.

Bacteria harvested at the end of the exponential phase of growth secreted more IpaC than those harvested earlier in this stage of growth. This increase in secretion of IpaC is likely to be due to the increase in the pool of cytoplasmic IpaC, since continuous expression of the *ipa* operon in the absence of secretion leads to accumulation of Ipa proteins within the cytoplasmic compartment. Indeed, a strain carrying an *ipaA-lacZ* transcriptional fusion contained approximately four times more β-galactosidase at the end than at the beginning of the exponential phase of growth. Likewise, accumulation of the components of the secretion machinery during bacterial growth is suggested by the increase in β-galactosidase activity produced by a strain carrying an *mxiD-lacZ* fusion. Therefore, bacteria taken throughout the exponential phase of growth appear to be equally competent to be activated by Congo red for secretion of Ipa proteins.

Congo red is a symmetrical molecule composed of two identical amino-substituted naphthalenesulfonate groups connected by azo linkages to a central biphenyl group. X-ray crystallographic studies have shown that the molecule is elongated, with a hydrophobic core and hydrophilic extremities, and can adopt different conformations due to the flexibility of the biphenyl spacer (15). Since both Evans blue and direct orange induced Ipa secretion, neither the nature of the charged substituents on the naphthalene groups nor the structure of the naphthalene groups appears to be important for activation. The amount of Evans blue and direct orange that was required to induce Ipa secretion was slightly higher than that of Congo red, which might be related to the presence of methyl substitutions on the central biphenyl group. In contrast, acid yellow-42, in which the biphenyl carries sulfonate substituents, had no activity. Likewise, neither the sulfonated naphthalene moiety alone (1-naphthylamine-4-sulfonic acid) nor a single phenyl group with a diazo linkage to a naphthalene group with charged substituents (naphthol blue black) was sufficient to induce secretion. This suggests that both the length and the hydrophobicity of the central region are essential for the interaction of the dye with its target. However, in the case of acid yellow-42, the anionic substituents on the diphenyl unit might also affect the respective positions of each phenyl group and induce a conformational change in the entire molecule which might not be planar. Further studies of the role of the biphenyl moiety, in the absence of charged groups, in activation of Ipa secretion could not be performed due to the insolubility of these compounds in PBS (3).

The mechanism of activation of Ipa secretion by Congo red can only be speculated on at this point. The hydrophobic core and hydrophilic extremities of Congo red, together with its conformational flexibility, allow this molecule to assume an optimum fit with target proteins, such as amyloid proteins implicated in neurodegenerative diseases (6, 26). Determination of the three-dimensional structure of a complex of Congo red and amyloid porcine insulin indicated that a single dye molecule binds to a pair of intact globular proteins by intercalating between antiparallel monomers (26). Also, both Congo red and Evans blue act as inhibitors of human immunodeficiency virus type 1 protease (5), and a model of the interactions of Congo red with the protease dimer has been proposed (15). In both cases, Congo red binds a hydrophobic pocket created at the interface of protein subunits within a complex. We have previously shown that IpaB and IpaD are required to block secretion in the absence of inducing signals and that a small proportion of both proteins is associated with the bacterial envelope (11). The phenotype of constitutive secretion of *ipaB* and *ipaD* mutants indicates that the inducer is not required for the activity of the secretion apparatus and

suggests that it does not act by modulating the size of the pore through which Ipa proteins are secreted. Therefore, it is conceivable that Congo red interacts with and destabilizes a protein complex which constitutes the cork of the secretion machinery. In this hypothesis, the inducer would not bind to a classical sensor protein to induce a conformational change leading to the opening of the secretion machinery.

Congo red has long been used to discriminate between invasive and noninvasive strains of *Shigella* (9, 18, 25). The red staining of colonies obtained on solid media containing Congo red is correlated with the activity of the Mxi-Spa machinery. The wild-type strain forms red colonies, whereas a virulence plasmid-cured strain and *mxi* and *spa* mutants form white colonies. The staining of colonies on plates and of bacterial pellets following incubation of bacteria in liquid media containing Congo red appears as the consequence of the binding of the dye to aggregates of proteins which are secreted by the type III secretion machinery of *Shigella*. Indeed, once in the extracellular medium, most of these proteins form aggregates which are heavily stained by Congo red (16). Thus, Congo red both activates the type III secretion machinery and binds to secreted proteins to produce the red color which is characteristic of wild-type isolates of *Shigella*.

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