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Individual chaperones required for Yop secretion by *Yersinia*

(targeting/translocation/virulence)

PIERRE WATTIAU, BÉATRICE BERNIER, PASCALE DESLÉE, THOMAS MICHIELS, AND GUY R. CORNELIS

Microbial Pathogenesis Unit, International Institute of Cellular and Molecular Pathology, and University of Louvain Medical School, B-1200 Brussels, Belgium

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ABSTRACT **Pathogenic yersiniae secrete anti-host proteins called Yops, by a recently discovered Sec-independent pathway. The Yops do not have a classical signal peptide at their N terminus and they are not processed during membrane translocation. The secretion domain is nevertheless contained in their N-terminal part but these domains do not resemble each other in the different Yops. We have previously shown that YopE secretion requires SycE, a 15-kDa acidic protein acting as a specific cytosolic chaperone. Here we show that the gene downstream from *yopH* encodes a 16-kDa acidic protein that binds to hybrid proteins made of the N-terminal part of YopH and either the bacterial alkaline phosphatase or the cholera toxin B subunit. Loss of this protein by mutagenesis led to accumulation of YopH in the cytoplasm and to a severe and selective reduction of YopH secretion. This protein thus behaves like the counterpart of SycE and we called it SycH. We also engineered a mutation in *lcrH*, the gene upstream from *yopB* and *yopD*, known to encode a 19-kDa acidic protein. Although this mutation was nonpolar, the mutant no longer secreted YopB and YopD. The product of *lcrH* could be immunoprecipitated together with cytoplasmic YopD. *lcrH* therefore seems to encode a YopD-specific chaperone, which we called SycD. Determination of the dependence of YopB on SycD requires further investigation. SycE, SycH, and SycD appear to be members of a new family of cytosolic chaperones required for Yop secretion.**

In bacteria, the Sec-dependent pathway, also referred to as the general secretory pathway (GSP), transfers proteins having an N-terminal signal sequence across the cytoplasmic membrane. The Sec apparatus consists of several integral membrane proteins and of a soluble ATPase (for a complete review, see ref. 1). Export of some preproteins requires SecB, a molecular chaperone whose primary function is to retard folding of the secretory precursors and possibly to pilot them to the export apparatus (2–5). In Gram-negative bacteria, the GSP releases proteins within the periplasmic space. It now appears that some bacteria have extended the GSP by various branches that translocate proteins across the outer membrane (for review, see ref. 1).

Two signal peptide-independent pathways are involved in the secretion of virulence proteins by pathogenic bacteria. The first pathway is used for α-hemolysin secretion by pathogenic strains of *Escherichia coli* (reviewed in ref. 6). The second type of secretory pathway, exemplified by the secretion of the 11 anti-host Yop proteins by yersiniae (for reviews see refs. 7–9), was recently encountered in various animal and plant pathogens (10).

The Yop secretory apparatus is encoded by about 20 genes from a 70-kb plasmid called pYV. Although Yops do not have classical N-terminal signal sequences, the secretion signal is nevertheless localized in the N terminus. There is no detectable similarity between the secretion domains of the various Yops, suggesting either that the signal is purely conformational or that each Yop possesses an individual targeting factor (11). We showed recently that an acidic homodimer, encoded by a gene neighboring *yopE*, is specifically involved in YopE secretion and is capable of binding the region of YopE that includes the secretion signal (12). We called this 130-amino acid cytosolic protein "SycE" for specific YopE chaperone and we proposed that its role is to guide the nascent YopE to the Yop-secretion machinery. If this is correct, other individual chaperones should be required for secretion of other Yops. We present evidence for two additional chaperones, SycH and SycD. They are specifically required for YopH and YopD secretion, respectively, and they bind to their corresponding target *in vitro*.

MATERIALS AND METHODS

Bacterial Strains. *Yersinia enterocolitica* KNG22703 (13) is an ampicillin-sensitive mutant of W22703 (14). pYV227 is their natural pYV plasmid. pGC1152 and pBM79 are pYV227 derivatives mutated in *yopH* and in *lcrV*, respectively (15, 16). The other yersiniae were *Yersinia pseudotuberculosis* YPIII (17) and *Yersinia pestis* EV76 (18). *E. coli* SM10 λ *pir*$^+$ was used to deliver mobilizable plasmids (19).

Genetic Constructs, DNA Sequencing, and Oligonucleotides. pPW37 was constructed by cloning a 240-bp DNA fragment internal to *sycH* synthesized using amplimers MIPA43 and MIPA44 in the *Sma* I site of pGCS82 (20), a suicide plasmid conferring resistance to gentamicin. *Y. enterocolitica* containing the pYV227::pPW37 cointegrates were selected on plates containing 35 μg of nalidixic acid per ml and 10 μg of gentamicin per ml. Plasmid pPW53 is a pBC19R (21) derivative carrying a 2680-bp *Hpa* II fragment from pYV227 containing *sycH* and the 3′ end of *yopH* downstream from the plac promoter.

To mutagenize *lcrH*, we first synthesized with amplimers MIPA121 and MIPA122 a *Bam*HI/*Hin*dIII DNA fragment including the complete gene flanked by 250 bp of DNA at each side. The fragment was cloned in the corresponding sites of pBC18R (21), yielding pPW64. This plasmid was linearized at the unique *Nco* I site in *lcrH* and subjected to exonuclease III digestion (Promega). Plasmid pPW64Δ16 carried an out-of-frame intragenic deletion leading to premature termination at codon 44. This mutant *lcrH* allele, called *lcrH16*, was first subcloned in pKNG101 (22) and exchanged with the wild-type allele of pYV227, giving pPW2269.

Plasmid pBB2 expresses *yopD* from the *yopE* promoter. It was constructed by subcloning a *Pst* I–*Bam*HI fragment of pYV227 (amplified with MIPA149 and MIPA150), filled at the *Bam*HI terminus, in the *Pst* I and the filled *Hin*dIII sites of pMS3 (23). The same *yopD* fragment was also cloned in pBluescript SK− (Stratagene), resulting in pBB1. In pBB1, *yopD* may be expressed from the ϕ10 promoter of bacteriophage T7. The pPW64 fragment containing *lcrH* was in turn

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Abbreviations: mAb, monoclonal antibody; CT-B, cholera toxin B subunit.

inserted in the *Hin*cII site of pBB1, giving pBB4. In pBB4, both *lcrH* and *yopD* are downstream from the ϕ10 promoter. Plasmid pPW70 was constructed by cloning both genes in the *Bgl* II site of pPW44, a mobilizable pSelect-1 derivative containing *yopE* with an engineered *Bgl* II site after the third codon (12).

Oligonucleotides MIPA43 (5'-TGTCTAGAGAATTTGCTACAGAGCT-3') and MIPA44 (5'-GACTGCAGAGCCAGTGATCATCTTT-3') derive from the *sycH* sequence. MIPA121 (5'-TTTGGATCCTAATGAATTATCTCAC-3') and MIPA122 (5'-CACAAGCTTGACCGACTCCAAT-3') derive from the *lcrGVH* sequence of *Y. pseudotuberculosis* (24). MIPA149 (5'-TTCTGCAGAAGGAGGAATACATATGACA-3') and MIPA150 (5'-CTGGATCCCTATCACCATAAGGCGTA-3') derive from the *yopBD* sequence of *Y. enterocolitica* (25).

The nucleotide sequence of *sycH* has been deposited in the GenBank data base (accession no. U08222).

Analysis of Yop Proteins. *yop* gene induction and Yop protein analysis were as described (11). For total cell protein analysis, 4×10^8 bacteria were solubilized in sample buffer and loaded on the gels. For analysis of the soluble cellular proteins, bacteria were disrupted by sonication and the crude extracts were centrifuged at $20,000 \times g$ for 1 hr. For analysis of secreted Yops, each lane of the SDS gels contained the Yops secreted by 3×10^8 bacteria.

Partial Purification of SycH and Purification of SycD. *E. coli* LK111 (pGP1-2)(pPW53) was grown and heat-induced in a 5-liter fermentor (New Brunswick Scientific) (26). Bacteria were harvested, resuspended in 8 ml of 50 mM phosphate/150 mM NaCl, pH 7.4 (PBS), and disrupted by sonication. The cell debris were pelleted by a 20-min centrifugation at $10,000 \times g$ and the supernatant was clarified by centrifugation at $100,000 \times g$ for 150 min. The supernatant was dialyzed against 10 mM Tris·HCl (pH 8.4) and loaded on a DEAE-Sephacel column (Pharmacia). Elution was performed by applying a 0–200 mM NaCl gradient in 100 ml of equilibration buffer and monitored by SDS/PAGE. Fractions containing SycH were pooled and stored at 4°C for a few hours, which resulted in the formation of a precipitate containing a large proportion of SycH. This precipitate, collected by centrifugation at $10,000 \times g$, was used to immunize a rabbit (12).

SycD was overproduced by *E. coli* LK111 (pGP1-2)(pPW64) as described for SycH and purified to homogeneity as described for SycE (12).

Immunodetection. Rabbit antisera raised against partially purified SycH or purified SycD were absorbed by adding lysates of KNG22703(pPW2237) or KNG227(pPW2269), respectively, and clarified by centrifugation. YopH was detected with rabbit polyclonal antiserum. YopD and YopE were detected with rat monoclonal antibodies (mAbs) 13A4 and 6G1, respectively (27). For Western blots, the absorbed sera were diluted 30-fold and the mAbs were diluted 250-fold in PBS supplemented with 5% lyophilized skimmed milk.

Protein Binding Assays. Binding of SycH on membrane-immobilized YopH and YopH-PhoA was performed as described (12). As a PhoA control, we used 5 μg of *E. coli* A19 alkaline phosphatase (Amersham). Anti-SycD immunoprecipitations were done as described by Kumamoto (28) except that protein G instead of protein A was covalently bound to agarose beads (Sigma). ELISA was performed by coating the plates with 100 μl of goat anti-cholera toxin serum (BioDesign, New York) diluted 100-fold. After overnight incubation at 4°C, the plates were blocked for 1 hr at room temperature with 200 μl of PBS supplemented with 10 mg of bovine serum albumin per ml and then washed three times with PBS supplemented with 0.1% Tween 20 (wt/vol) (PBS/Tween). The plates were then incubated for 2 hr at room temperature with 100 μl of *Y. enterocolitica* soluble extracts (about 800 μg of total protein per ml) containing the relevant chaperone and target. The plates were again washed three times with PBS/Tween and incubated for 2 hr at room temperature with serial dilutions of absorbed rabbit anti-SycH serum. After three additional washes, 100 μl of horseradish peroxidase (HRP)-conjugated swine anti-rabbit immunoglobulin (γ-chain-specific immunoglobulin) diluted 300-fold (Dakopatts, Glostrup, Denmark) was added. The plates were then washed again and HRP-labeled antibodies were revealed with 100 μl of 0.4 μg of *o*-phenylenediamine per ml (Sigma) in 82 mM phosphate/43 mM citrate buffer, pH 5, containing 0.01% H_2O_2. The reaction was stopped with 50 μl of 2 M H_2SO_4 and read at 490 nm in an automatic ELISA reader (Bio-Rad).

RESULTS

Localization and Nucleotide Sequence of the Gene Encoding the SycH Chaperone. When sequencing DNA downstream from *yopH* (29), we identified an open reading frame (ORF) oriented in the opposite direction. It encodes a putative protein of 141 residues with a calculated molecular mass of 15,719 Da, an isoelectric point (pI) of 4.88, and no classical N-terminal signal sequence. This protein appeared as a likely candidate for SycH function. According to FAST and BLAST homology search programs (30, 31), the amino acid sequence of SycH is not similar to that of SycE. However, they resemble each other in terms of predicted physicochemical properties and secondary structures. In particular, the hydrophobic moment plots, which were almost identical, exhibited two major peaks at the C-terminal end (Fig. 1*A*). The amino acid sequence of the last peak in both proteins contains the perfectly conserved motif Leu-Xaa$_6$-Asn-Xaa$_6$-Leu-Xaa$_6$-Leu-Xaa$_6$-Leu (Fig. 1*B*). This motif, resembling a leucine zipper, begins at residue 91 of SycH and at residue 89 of SycE. The secondary structure prediction gives a high probability of α-helix structure for the last 20 residues of this motif. When drawn in α-helical wheel projection, this domain of the two proteins displayed an even more striking similarity (Fig. 1*C*).

When compared to data bases, the SycH protein sequence appeared to be weakly similar to ORF-U (19% total similarity), a hypothetical 17-kDa protein with a pI of 4.15, identified in enteropathogenic *E. coli* (EPEC) and in *Citrobacter freundii* (33, 34). Interestingly, the C-terminal part of ORF-U contains a putative amphiphilic α-helix motif starting at residue 95 and containing a degenerate leucine repeat (Leu-Xaa$_6$-Asp-Xaa$_6$-Leu-Xaa$_6$-Val-Xaa$_6$-Leu).

Construction and Analysis of a SycH-Deficient *Y. enterocolitica* Strain. The *sycH* gene of *Y. enterocolitica* W22703 (pYV227) was mutagenized by integration of pPW37, a suicide plasmid containing 240 bp internal to *sycH*. The resulting mutant, W22703(pPW2237), secreted all Yops except YopH (Fig. 2). In contrast, bacteria lacking SycH contained much more cytosolic YopH than the parental strain (Fig. 2). The mutation could be complemented in trans by the cloned *sycH* gene present on pPW53 and transcribed from a vector promoter. Thus, the mutation specifically blocked YopH secretion. Unlike the *sycE* mutant (12), the *sycH* mutant accumulated the partner protein in the cytoplasm.

Identification of SycH in *Y. pestis* and *Y. pseudotuberculosis*. SycH was overproduced in *E. coli* LK111 and partially purified to raise antibodies. By Western blotting, the antiserum recognized a soluble 17-kDa cellular protein missing in the *sycH* mutant. It was also detected in extracts of *Y. pseudotuberculosis* and *Y. pestis* (data not shown).

Binding of SycH to the N-Terminal Part of YopH. To determine whether SycH is able to bind YopH, we incubated a crude extract of *E. coli* producing SycH with secreted Yops immobilized on a nitrocellulose membrane after SDS/PAGE (12). We included in the experiment a culture supernatant of *Y. enterocolitica* W22703(pTM190) that secretes not only all

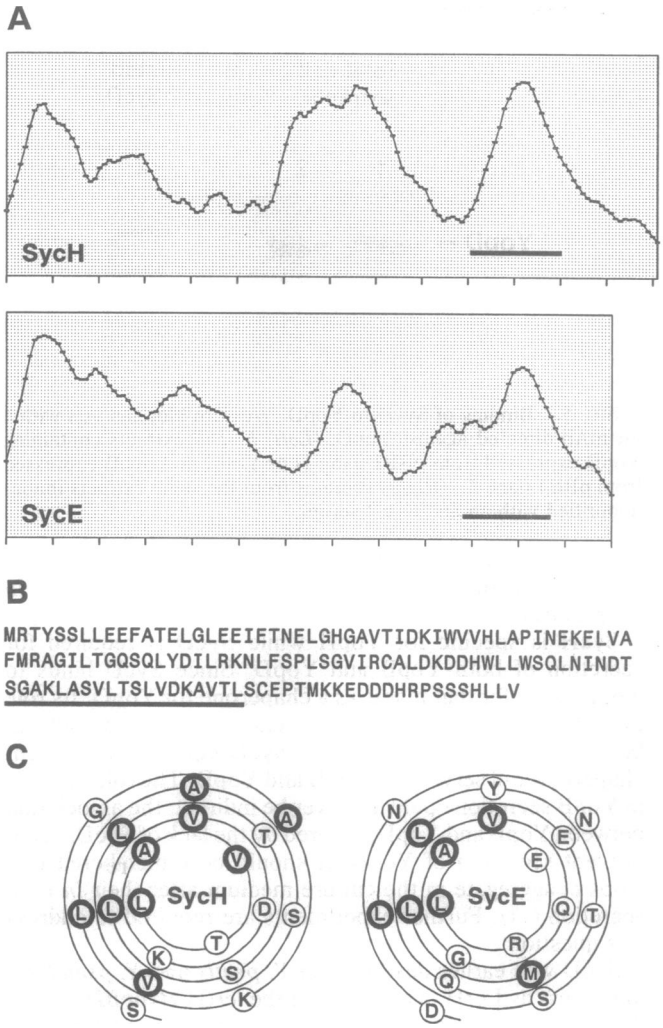

FIG. 1. Similarity between SycH and SycE (13). (*A*) Hydrophobic moment plots (32). The underlined peak in the SycH plot corresponds to residues underlined in the sequence. (*B*) Sequence of SycH. The underlined residues correspond to a putative amphiphilic α-helix. (*C*) α-Helical wheel projection of the residues forming the underlined peaks shown in *A*. Thick circles correspond to hydrophobic residues, and thin circles correspond to hydrophilic residues.

of the Yops but also a hybrid YopH-PhoA protein consisting of the first 69 amino acids of YopH fused to the mature part of the *E. coli* alkaline phosphatase (EC 3.1.3.1) (35). Binding of SycH was immunodetected by using the anti-SycH serum. We observed binding of SycH not only to YopH but also to the YopH-PhoA hybrid and not to PhoA (Fig. 3).

To detect binding of SycH to YopH derivatives under different conditions, we turned to an ELISA approach. As a target protein, we selected a hybrid consisting of the N-terminal domain of YopH fused to the cholera toxin B subunit (CT-B). Plasmid pMS44 encoding a hybrid made of the first 125 N-terminal residues of YopH fused to the mature part of CT-B (36) was introduced in the wild-type *Y. enterocolitica* W22703(pYV227) strain and in its SycH$^-$ derivative. As expected, the YopH-CT-B hybrid was efficiently secreted by SycH$^+$ bacteria, whereas it remained blocked inside SycH$^-$ bacteria (not shown). To investigate the binding of SycH to YopH-CT-B, we coated microtiter plates with goat anti-CT-B immunoglobulin. We then incubated the plates with the soluble cellular proteins from SycH$^+$ or SycH$^-$ yersiniae, both producing the hybrid YopH-CT-B. Binding of SycH was revealed with the rabbit anti-SycH serum followed by peroxidase-conjugated anti-rabbit immunoglobulin. The signal

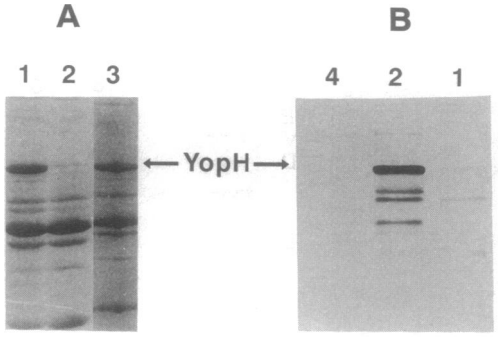

FIG. 2. Phenotype of the *sycH* mutant. (*A*) SDS/PAGE analysis of the Yops secreted by wild-type *Y. enterocolitica* W22703-(pYV227)$^+$ (lane 1), *sycH* mutant W22703(pPW2237) (lane 2), and complemented *sycH* mutant W22703(pPW2237)(pPW53) (lane 3). The *sycH* mutant was not complemented by the vector pBC18R (not shown). (*B*) Western-blot analysis of YopH in the total cell proteins from the wild-type strain *Y. enterocolitica* W22703(pYV227) (lane 1), *sycH* mutant W22703(pPW2237) (lane 2), and *yopH* mutant W22703(pGC1152) (lane 4) (15).

was significantly higher with extracts from YopH-CT-B$^+$, SycH$^+$ bacteria than with extracts from YopH-CT-B$^+$, SycH$^-$ bacteria (not shown). Extracts from wild-type bacteria, producing no YopH-CT-B, gave no signal, confirming the specificity of the anti-CT-B serum and of the reaction. Thus, although the cytoplasm of SycH$^+$ bacteria contained much less YopH-CT-B than that of SycH$^-$ bacteria, it gave a stronger signal, revealing clear binding between SycH and YopH.

Identification of the YopD Chaperone. YopD, the most abundant protein secreted by *Y. enterocolitica*, is encoded by the *lcrGVHyopBD* operon (16, 24, 37). The product of *lcrH* is a 19-kDa protein with a predicted pI of 4.53 (37). It has been proposed to act as a negative genetic regulator (24, 38) but it was never shown to bind DNA. On the basis of its gene location, size, and pI, we considered that LcrH could be a specific chaperone serving YopB and/or YopD. To test this, we constructed KNG22703(pPW2269), a *lcrH* mutant of *Y. enterocolitica* KNG22703(pYV227). The mutant, carrying the *lcrH16* allele, was thermosensitive for growth but, when incubated at 37°C in the absence of Ca^{2+}, it secreted all Yops, except YopB and YopD (Fig. 4). It did not secrete the Yops at low temperature or in the presence of Ca^{2+} (data not shown). To ensure that the lack of YopD secretion did not result from a polar effect of *lcrH16* on the downstream *yopD*

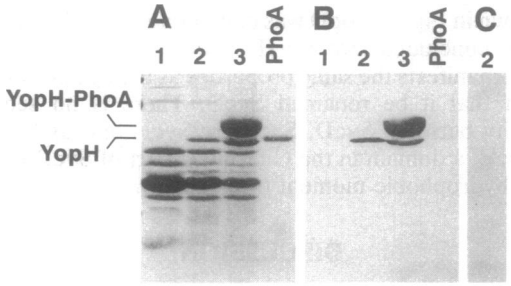

FIG. 3. Binding of SycH to YopH and to a YopH-PhoA hybrid. (*A*) SDS/PAGE of the proteins secreted by *yopH* mutant W22703-(pGC1152) (15) (lane 1), by wild-type W22703(pYV227) (lane 2), and by W22703(pYV227)(pTM190) secreting intact YopH and a hybrid YopH-PhoA (lane 3) (35). Five micrograms of purified *E. coli* alkaline phosphatase was added as a control (lane PhoA). (*B*) Western-blot analysis of the same samples after incubation with a cleared extract of temperature-induced *E. coli* LK111(pGP1-2)(pPW53) containing SycH and followed by immunodetection with the anti-SycH serum. (*C*) Antiserum control. Yop proteins were probed directly with the anti-SycH serum (lane 2 as in *A* and *B*).

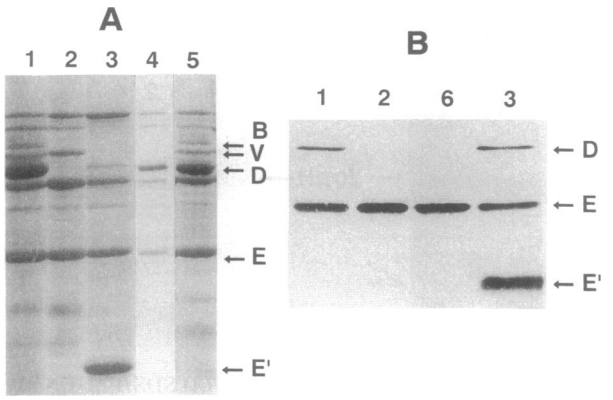

FIG. 4. Phenotype of the *lcrH* mutant. (*A*) SDS/PAGE analysis of the Yops secreted by wild-type *Y. enterocolitica* W22703(pYV)$^+$ (lane 1), by *lcrH* mutant W22703(pPW2269) (lane 2), by *lcrH* mutant W22703(pPW2269)(pBB2) overproducing YopD from the *yopE* promoter (lane 3), by a *lcrV* polar mutant W22703(pBM79)(pPW70) complemented with both *lcrH* and *yopD* (lane 4) (16), and by *lcrH* mutant W22703(pPW2269)(pPW64) complemented with a cloned *lcrH* gene (lane 5). (*B*) Western-blot analysis of soluble cellular proteins with anti-YopE and anti-YopD mAbs. Lanes 1–3 are as in *A*. Lane 6 is the *lcrV* polar mutant W22703(pBM79) with no additional plasmid. Yops are identified by their code letter. E′ represents a truncated form of YopE (130 amino acids) expressed from pBB2.

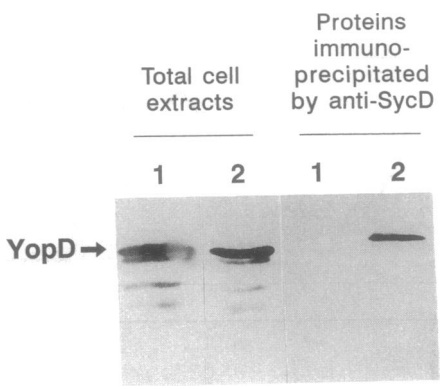

FIG. 5. Binding of SycD to YopD. Western-blot analysis with a rat mAb directed against YopD. (*Left*) *E. coli* extracts containing YopD expressed from pBB1 (lane 1) or YopD and SycD expressed from pBB4 (lane 2). (*Right*) Proteins from the same extracts immunopurified with an anti-SycD serum.

gene, we introduced plasmid pBB2, which contains *yopD* cloned behind the strong *yopE* promoter. In spite of the fact that YopD was detected inside bacteria harboring pBB2, YopD secretion was not restored (Fig. 4). In contrast, secretion was restored by introducing plasmid pPW64, which contains *lcrH* expressed from the plac promoter. The *lcrH16* mutation is thus nonpolar and LcrH is a factor specifically required for secretion of YopB and YopD. YopD was also secreted by *Y. enterocolitica* W22703(pBM79)(pPW70) carrying a *lcrV* polar mutation as well as *yopD* and *lcrH* cloned downstream from the *yopE* promoter. YopD could not be detected inside the cells of the LcrH$^-$ bacteria containing only the wild-type *yopD* gene, which suggested that YopD was rapidly degraded in the absence of LcrH (Fig. 4).

LcrH Binds to YopD. To detect a possible association between LcrH and YopD, we produced either YopD or YopD plus LcrH in *E. coli* using the T7 expression system (26). Both soluble extracts were incubated with rabbit anti-LcrH antibodies and then with protein G covalently bound to agarose beads (Sigma). The immunoabsorbed proteins were analyzed by Western blot with the anti-YopD mAb 13A4 (27). As shown in Fig. 5, YopD was coimmunopurified with LcrH, and we concluded that LcrH specifically binds to YopD. Since it manifests the same properties as the Syc proteins, we suggest that it be renamed SycD. There is no sequence similarity between SycD, SycE, or SycH but, as for SycH and SycE, a domain in the C-terminal part of SycD displays a high hydrophobic moment (not shown).

DISCUSSION

We have previously shown that secretion of YopE specifically involves a 15-kDa cytosolic chaperone called SycE and we hypothesized that this chaperone would act as a targeting factor (12). If this interpretation is correct, one should be able to identify other such chaperones in the Yop secretion system. We show here that secretion of YopH, YopB, and YopD also requires cytosolic proteins. By analogy with SycE, we called these proteins SycH and SycD. The three chaperones share several common properties: the pI is very acidic (4.55 for SycE, 4.88 for SycH, and 4.53 for SycD), the size is on the order of 15–20 kDa, and they are encoded by genes adjacent to the gene encoding the Yop protein they serve. They appear to constitute a new family of protein chaperones.

SycH is specific for YopH while SycD is required for secretion of both YopB and YopD. Since SycD binds to YopD, we infer that it acts as a chaperone for YopD. Its role in YopB secretion was not investigated in this study but can be interpreted in several ways. SycD could be a bivalent chaperone and serve both YopB and YopD. The role of SycD in YopB secretion could however be indirect: the association between YopB and YopD, favored by the lack of SycD, could prevent secretion of YopB. It should be remembered that Yops coaggregate in the culture medium after their *in vitro* secretion (11). Further experiments are required to address this question.

SycD was earlier described in *Y. pestis* and *Y. pseudotuberculosis* as LcrH, a regulator of the *lcrGVHyopBD* operon (24, 38). Our results demonstrate that SycD is required for YopD secretion rather than for transcription but do not rule out an additional indirect effect on transcription of the *lcrGVHyopBD* operon. This has been shown for other secretory mutations, in particular in the *virB* locus (refs. 39–42).

Yop secretion probably does not involve the recognition of a common N-terminal sequence but this lack of common address sequence could be compensated by the existence of individual chaperones. Such ancillary proteins could be the hallmark of the Yop secretion pathway. Since similar pathways are now encountered in several animal or plant pathogens, many chaperones could be identified in a near future. In this respect, SycH appears to be related to ORF-U, a hypothetical protein of EPECs and *C. freundii* (33, 34). Its function is unknown, but similarities with the Syc proteins suggest a related function. It is encoded by a gene lying close to *eae*, which encodes an outer-membrane protein (33). The presence of this protein suggests the existence of a secretion system related to the Yop secretion system in EPECs and *C. freundii*.

We have shown previously that SycE binds the 128 N-terminal residues of its target YopE. Here we show that SycH binds the shortest YopH-PhoA hybrid that can be efficiently secreted, a YopH-PhoA hybrid made of only 69 residues of YopH (35). It is therefore tempting to assign to Syc proteins a role in signal recognition. If the role of the Syc proteins is indeed to recognize the Yop secretion signal or a part of it and to pilot the nascent protein to the secretion machinery, the sequence of the Syc proteins should consist of at least two distinct domains: a variable domain, involved in Yop recognition, and a constant domain, required for the specific

interaction with the secretory apparatus. SycE and SycH are very weakly related in terms of amino acid sequence but they display similar hydrophobic moment plots. The C-terminal part of SycE and SycH also contains a conserved motif resembling the so-called "leucine repeat" motif. This motif, corresponding to an α-helix protein structure where most of the hydrophobic residues, essentially leucines, are disposed on the same side of the helix, could represent the constant domain of the Syc chaperones. The C terminus of SycD also contains a potential amphiphilic α-helix but it does not include a leucine repeat. Leucine repeats are thought to be involved in protein–protein associations but have so far been described for only DNA-binding proteins (43–45). In the Syc proteins, they could interact with the secretory apparatus but they could also be involved in the dimerization we observed for SycE (12). The role of the C-terminal amphiphilic α-helix motif in the Syc chaperones thus awaits mutational analysis.

Rather than being targeting factors, the Syc proteins could be anti-folding factors or they could protect intracellular Yops from degradation. The latter hypothesis can be ruled out for SycH and possibly SycE but not for SycD. YopH indeed accumulates in the absence of SycH, whereas the amount of intracellular YopE is not altered in the absence of SycE (12). By contrast, in the absence of SycD, YopD was not detectable inside the bacterial cell unless it was overproduced. YopD seems thus to be rapidly degraded when its chaperone is absent.

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