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Analysis of Expression and Thermoregulation of the *Yersinia pseudotuberculosis inv* Gene with Hybrid Proteins

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A series of translational fusions between the *Yersinia pseudotuberculosis inv* locus and *lacZ* was constructed. Each Lac$^+$ fusion strain expressed a hybrid protein containing invasin, the product of the *inv* locus, at its amino-terminal end. Analysis of these gene fusions allowed determination of the direction of translation of the *inv* gene. Previous studies of *Y. pseudotuberculosis* invasion have shown that entry into animal cells is temperature regulated. It is shown here that control of expression of the *inv* gene is also temperature regulated. *phoA* gene fusions to *inv*, when present in *Y. pseudotuberculosis*, were expressed at lower levels when bacteria were grown at 37°C rather than at 28°C. Similar fusions, in contrast, were regulated in a temperature-independent fashion in *Escherichia coli*, as was the wild-type *inv* gene. This implies that *Y. pseudotuberculosis* has chromosomally encoded *trans*-acting functions that normally thermoregulate expression of *inv*.

Yersinia pseudotuberculosis is a facultative intracellular enteric bacterium (1–3, 16). Pathogenesis by this enteroinvasive microorganism is initiated by entry into epithelial layers followed by eventual localization and growth of bacteria in regional lymph nodes, the liver, and the spleen (5, 18). The first step in the process, entry into epithelial cells, can be reproduced in tissue culture models (1, 2, 8), allowing straightforward molecular analysis. The bacterium is able to enter HeLa or HEp-2 cells efficiently in culture, with as many as 20% of the added bacteria being internalized by the monolayer cells (2, 10). Once inside the cultured cells, the bacteria are found localized within membrane-bound vacuoles, where they are protected from the action of aminoglycoside antibiotics (2, 8).

We have been investigating the mechanism of *Y. pseudotuberculosis* entry into HEp-2 cells. Our approach has been to use molecular clones isolated from *Y. pseudotuberculosis* that convert *Escherichia coli* K-12 into an organism able to penetrate monolayer cells (8). We have determined that a single 3.2-kilobase gene from *Y. pseudotuberculosis*, called *inv*, is sufficient to confer this phenotype. Molecular clones of *inv* were originally detected by isolating *E. coli* strains that were protected from gentamicin treatment after establishing an *inv*-mediated intracellular niche (8). *E. coli* strains harboring plasmids encoding *inv* express a 103-kilodalton protein, exposed on the surfaces of maxicells, that apparently mediates the entry process (9). This protein, called invasin, is necessary for the parental *Yersinia* organism to enter cells efficiently, since *Y. pseudotuberculosis inv* mutants are unable to penetrate HEp-2 cells efficiently (9).

In the present study, we used gene fusions between *inv* and the *E. coli lacZ* gene to study invasin. This allowed us to determine the direction of translation of this gene, as well as analyze its temperature-dependent regulation.

MATERIALS AND METHODS

Bacterial strains, media, and chemicals. The bacterial strains used are described in Table 1. The bacteriological media used were as described previously (8, 13). HEp-2 cells were maintained throughout in RPMI 1640 medium containing 5% fetal bovine serum. 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside was purchased from Clontech, and 5-bromo-4-chloro-3-indolylphosphate was obtained from Sigma Chemical Co. [^{35}S]methionine was purchased from New England Nuclear Corp., and molecular weight standards for polyacrylamide gels were obtained from Bio-Rad Laboratories. Assays for entry of bacteria into HEp-2 cells were performed in Falcon 3047 24-well microtiter dishes. Restriction enzyme digestions were in buffers suggested by the manufacturer (New England BioLabs, Inc.).

Isolation of gene fusions to *lacZ*. Random insertions of mini Mu d*lac*II1734 were isolated in pRI203 as previously described (6). Lac$^+$ colonies (detected on 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside plates) that were the result of Mu d*lac*II insertion into pRI203 were then assayed for their ability to enter HEp-2 cells (8). Insertions that caused an Inv$^-$ phenotype were saved and analyzed further. Sites of insertion were mapped by restriction enzyme digestions, and the production and size of hybrid proteins were determined by immunoprecipitations of extracts from cells biosynthetically labeled with [^{35}S]methionine.

pRI211 and pRI212 were constructed by digesting Φ(*inv-lacZ*)*2*(Hyb)(pRI211) and Φ(*inv-lacZ*)*5*(Hyb)(pRI212) with *Hin*dIII and *Bgl*II, followed by insertion of the fragments spanning each gene fusion into pBR325 digested with *Hin*dIII and *Bam*HI. These subclonings allowed deletion of any Mu sequences located 3′ downstream from the gene fusion but left the hybrid proteins intact.

Isolation of gene fusions to *phoA*. Insertions of Tn*5* IS*50*$_L$::*phoA* (Tn*phoA*) were isolated on plasmid pRI233 (*inv*$^+$) as described previously, by using λ *b*221 *rex*::Tn*5*-*phoA* *c*I857 *P*am80 as the transposon donor (12). Insertions isolated on pRI233 that conferred kanamycin resistance and were Pho$^+$ (as determined by plating on 5-bromo-4-chloro-3-indolylphosphate-containing media) were then assayed for the ability to enter HEp-2 cells to screen for strains harboring fusions to the *inv* gene. Plasmids were isolated from such strains, and the sites of the transposon insertions were mapped physically by restriction endonuclease digestion.

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TABLE 1. Bacterial strains and plasmids

Bacterial strain or plasmid	Description or genotype	Source or reference
E. coli		
MC1000	F⁻ Δ(*ara-leu*) Δ(*lac*)X74 *rpsL galE galK*	6
M8820Mu	F⁻ Δ (*ara-leu*) Δ(*proAB lac*)XIII *rpsL* (Mu)	6
M8820Mucts	Same as M8820Mu except with Mu cts instead of Mu	6
SE5000	F⁻ *recA56 araD* Δ(*lac*)U169 *rpsL*	S. Emr
MALII1734	F⁻ *malK* or *malT*::Mu *araD* Δ(*ara leu*) Δ(*proA lac*) *rpsL* Mu DII1734 (Kan^r)	6
SM10(λ *pir*)	Tra$^+$ RP4	R. Simons
Y. pseudotuberculosis		
YPIII(P⁻)	Cured of plasmid pIB1	1
YPh10	YPIII(P⁻) *inv*$^+$-*amp-inv-1*::Tn*phoA*	This study
YPh70	YPIII(P⁻) *inv*$^+$-*amp-inv-7*::Tn*phoA*	This study
YPh110	YPIII(P⁻) *inv-1*::Tn*phoA*	This study
YPh170	YPIII(P⁻) *inv-7*::Tn*phoA*	This study
Plasmids		
pRI203	pBR325 *inv*$^+$	9
pRI203 (Hyb)	pRI203 derivatives containing random insertions of mini Mu d*lac*II1734	This study
pRI211	pBR325 *lac-41*(Hyb) deleted for Mu sequences	This study
pRI212	pBR325 *lac-41*(Hyb) deleted for Mu sequences	This study
pRI203.51	pRI203 *inv-51*::Tn*1000*	This study
pRI203.61	pRI203 *inv-61*::Tn*1000*	This study
pRI203.72	pRI203 *inv-72*::Tn*1000*	This study
pRI203.93	pRI203 *inv-93*::Tn*1000*	This study
pRI203.169	pRI203 *inv-169*::Tn*1000*	This study
pRI203.201	pRI203 *inv-201*::Tn*1000*	This study
pRI233	Amp^r *ori*R6K *ori*TRP4 *inv*$^+$	9
pIPh1	pRI233 *inv-1*::Tn*phoA*	This study
pIPh7	pRI233 *inv-7*::Tn*phoA*	This study

Approximately 25% of the *inv* insertions (11 of 40) yielded a Pho$^+$ phenotype, consistent with the frequency predicted for insertion of *phoA* in a gene encoding a membrane protein (12).

Isolation of *inv-phoA* insertions on the *Y. pseudotuberculosis* chromosome. In a similar fashion to that described previously (9, 13a), the pRI233 *inv*::*phoA* insertions were introduced onto the *Y. pseudotuberculosis* chromosome. *E. coli* SM10(λ *pir*), which encodes the R6K replication protein pi and the transfer functions of RP4, was used to stably maintain and mobilize pRI233:Tn*phoA* derivatives. Matings between this donor strain and *Y. pseudotuberculosis* YPIII(P⁻) were performed as described previously (11), selecting for one of the drug resistance determinants on this plasmid. Inasmuch as *Y. pseudotuberculosis* does not express the pi protein and cannot replicate pRI233 derivatives, antibiotic-resistant strains that arise are the result of plasmid integration into the chromosome via the homology provided by the *inv* locus. Amp^s segregants haploid for the *inv* locus were isolated from these merodiploids (see Fig. 4). Segregants were analyzed by Southern blot hybridization to determine whether the chromosome of these recombinants had the appropriate genetic structure.

Production of maxicells, in vitro translation, and immunoprecipitation techniques. Maxicells were produced from SE5000 by using standard techniques (16). Biosynthetic labeling with [^{35}S]methionine and immunoprecipitations of samples boiled in sodium dodecyl sulfate (SDS) were as previously described (10). S30 extracts were produced previously (9), and the coupled transcription-translation reactions were performed as described by Brusilow et al. (4).

Assays for binding and entry of bacteria into animal cells. Bacteria were assayed for binding to animal cells by incubation of HEp-2 monolayers, seeded at a concentration of 10^5 cells per microtiter well, with 10^7 bacteria from a culture grown to an A_{550} of 0.6. The bacteria were pelleted onto the monolayers for 10 min at 500 × *g* and allowed to incubate on ice for 90 min. Nonadherent bacteria were removed by being washed 10 times with ice-cold phosphate-buffered saline. The bacteria were released from the monolayers with 1% Triton X-100, and viable counts were determined on L agar plates.

Bacteria were assayed for entry into HEp-2 cells as previously described (8). Internalization of bacteria by HEp-2 cells was quantitated by survival after 1 h of gentamicin treatment at 36°C.

Enzyme assays. Determinations of β-galactosidase (13) and alkaline phosphatase (12) activities were performed as described previously.

RESULTS

Direction of translation of the *inv* locus. The primary translation product of the *inv* locus is a 103-kilodalton peptide, called invasin, that apparently saturates the coding capacity of this locus (9). To initiate a study of the expression of this gene, we wanted to determine its direction of translation by using *E. coli* strains harboring molecular clones of *inv*. A series of hybrid proteins was constructed by isolating gene fusions between the *inv* region and the *E. coli lacZ* gene encoding β-galactosidase. The direction of expression of each active gene fusion was determined on the basis of the known restriction map of *lacZ* (6), and the resulting hybrid proteins were analyzed on SDS-polyacrylamide gels.

Random insertions of mini Mu d*lac*II1734 were isolated on a plasmid encoding an active *inv* locus (6; Materials and Methods). Translational fusions between *inv* and *lacZ* were identified by isolation of insertions that yielded a Lac$^+$ phenotype on indicator medium, followed by screening of candidates for loss of the ability to penetrate HEp-2 cells (Materials and Methods). This phenotype indicated that translational fusions had been isolated that disrupted the *inv* locus and generated an active β-galactosidase, presumably because the *lacZ* gene was in the same reading frame as *inv*. Figure 1 displays the site of insertion and direction of translation of the *lac* gene for each of the fusions, as determined by restriction enzyme analysis. All of the Lac$^+$ insertions that eliminated the ability to penetrate animal cells were located in the previously defined *inv* gene. Furthermore, the direction of translation of each gene fusion was identical (5′ end at the left of the map in Fig. 1), in agreement with our previous data that *inv* probably is a single gene (8, 9).

The hybrid proteins generated by the gene fusions were analyzed by immunoprecipitation of biosynthetically labeled

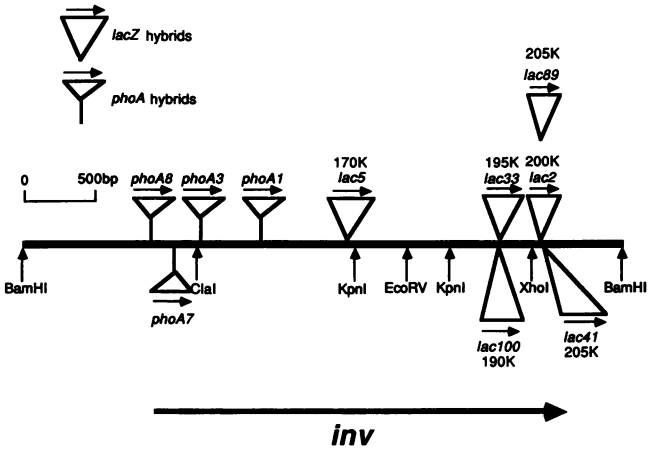

FIG. 1. Site of insertion, orientation, and sizes of hybrid proteins produced by *lacZ* and *phoA* fusions. Gene fusions between the *inv* region and the *lacZ* gene encoding β-galactosidase were isolated after random insertion of the mini Mu d*lac*II (6; Materials and Methods). *inv-phoA* fusions were constructed by using Tn*phoA* (12; Materials and Methods). The triangles represent insertion sites of mini Mu d*lac* or Tn*phoA* that eliminate the ability of the bacterium to enter animal cells and also result in a Lac$^+$ or Pho$^+$ phenotype on indicator medium (Materials and Methods). Shown above each triangle is the strain number, the direction of translation of each gene fusion, and, for *lacZ* fusions, the sizes of hybrid proteins that resulted from each fusion. Direction of translation was determined by restriction digestions based on the known map of *lacZ* or Tn*phoA*. Sizes of *lacZ* hybrid proteins were determined by anti-β-galactosidase immunoprecipitation of biosynthetically radiolabeled proteins from growing bacterial strains, followed by analysis on SDS-polyacrylamide gels (Materials and Methods; Fig. 2). The arrow labeled *inv* is the orientation of translation of this gene as predicted by these data. bp, Base pairs; 205K, 205,000-molecular-weight protein.

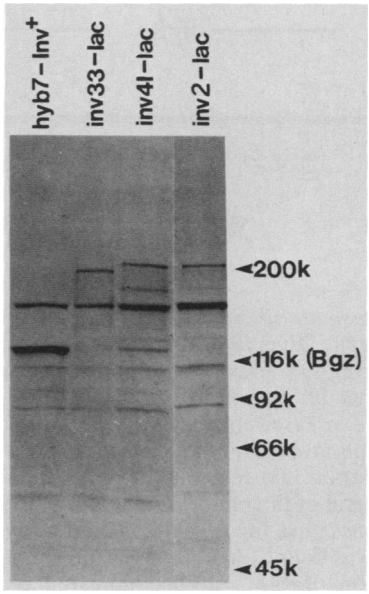

FIG. 2. Large hybrid proteins generated by gene fusions. Displayed is the SDS-polyacrylamide gel analysis of the products of four *lacZ* gene fusions generated as described in the legend to Fig. 1. *E. coli* MM8820 (Mu$^+$) containing the described plasmids was grown at 30°C, labeled with [^{35}S]methionine, and subjected to immunoprecipitation with anti-β-galactosidase as described in Materials and Methods. Shown are plasmids containing *hyb-7*, a random gene fusion to sequences in pBR325, and three of the fusions described in Fig. 1 that disrupt the *inv* gene. Molecular weights were determined by staining of the gel with Coomassie blue to detect the following protein size standards: myosin, 200,000 (200k); *E. coli* β-galactosidase (Bgz), 116,000 (116k); phosphorylase b, 92,500 (92k); bovine serum albumin, 66,500 (66k); ovalbumin, 45,000 (45k).

extracts with anti-β-galactosidase, followed by SDS-polyacrylamide gel electrophoresis (Fig. 1 and 2). The results supported the restriction enzyme analysis and showed that the sizes of the hybrid proteins correlated with the positions of insertion in the *inv* locus (Fig. 1 and 2). This also indicates that each Lac$^+$ colony had placed *lacZ* in the same reading frame as the *inv* gene.

To gain independent support for our determination of the direction of translation, a truncated peptide analysis was performed with S30 extracts from *Y. pseudotuberculosis* that could express proteins from exogenously added plasmid DNA. The plasmids contained Tn*1000* insertions at different locations within the *inv* gene to disrupt the synthesis of invasin. Each plasmid produced a truncated major translation product (Table 2; Fig. 3) that directly corresponded to the site of the Tn*1000* insertion and was consistent with the direction of translation predicted by the *lacZ* gene fusions (Fig. 1). For example, an insertion mutation that was mapped by restriction analysis to the 3′ end of the gene resulted in a major translation product having an apparent molecular weight of 90,000 in the S30 system (Table 2, insertion 51). An insertion near the 5′ end of the gene, on the other hand, resulted in the major translation product being only 12 kilodaltons (insertion 169). We conclude from this analysis that the direction of translation must be as described in Fig. 1. This direction of translation is identical to that predicted from the major open reading frame found when we sequenced the *inv* region previously, thus giving independent verification of the validity of this analysis (9).

Temperature regulation of gene fusions. The efficiency of *Y. pseudotuberculosis* entry into cultured mammalian cells has been reported to be dependent upon the temperature at which the bacteria are cultured (1). Table 3 illustrates this

TABLE 2. Size of invasin and its direction of translation as determined by hybrid proteins[a]

Tn*1000* insertion[b]	Site of insertion (kb)[c]	Mol wt of truncated product[d]
41	pBR325	108,000
61	3.95	97,000
51	3.80	90,000
201	3.42	77,000
93	2.15	40,000
72	1.80	30,000
169	1.50	12,000

[a] Plasmids containing the Tn*1000* insertions described in Fig. 3 were introduced into a coupled transcription-translation extract made from *Y. pseudotuberculosis* (Materials and Methods). The [^{35}S]methionine-labeled in vitro synthesized proteins (4) were analyzed on polyacrylamide gels (11) after allowance of 60 min for translation at 30°C, and the molecular weights were determined relative to Coomassie blue-stained protein standards.

[b] These Tn*1000* insertions are depicted in Fig. 3.

[c] The distance of each insertion from the leftmost *Bam*HI site is shown in Fig. 3.

[d] Molecular weights were determined from polyacrylamide gels by using the following proteins as size standards: myosin, 200,000; *E. coli* β-galactosidase, 116,000; phosphorylase *b*, 92,500; bovine serum albumin, 66,500; ovalbumin, 45,000; carbonic anhydrase, 31,000; soybean trypsin inhibitor, 21,500; lysozyme, 12,500.

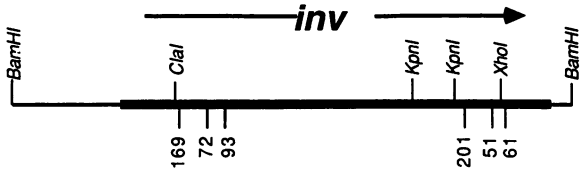

FIG. 3. Diagram of Tn*1000* insertions showing direction of translation.

property. *Y. pseudotuberculosis* strains grown at 28°C entered 10 times as efficiently as bacteria grown at 37°C; *E. coli* strains harboring *inv*, in contrast, showed no such temperature dependence for penetration (Table 3). This suggests that the *inv* locus expressed in *E. coli* is no longer controlled by the same temperature-dependent regulatory circuit found in the parental strain. To test this hypothesis, *E. coli* strains harboring several of the plasmids encoding the *inv-lacZ* gene fusions were assayed for β-galactosidase activity at 28 and 37°C. None was found to be temperature regulated in any marked fashion (Table 4). This indicated either that our clones contained insufficient information at their 5′ ends to be sensitive to temperature regulation or that the factors that control temperature regulation are found only in *Y. pseudotuberculosis*. Inasmuch as more than 1,000 base pairs are located 5′ upstream from the end of *inv* in these clones (9), it seems likely that there are *trans*-acting factors present in *Y. pseudotuberculosis*, but not in *E. coli*, that regulate expression of *inv*.

To test whether thermal regulation of cell penetration was due to control of *inv* gene expression, we measured the expression of this gene in *Y. pseudotuberculosis*. We constructed a series of gene fusions between *inv* and *phoA*, encoding the easily assayable *E. coli* alkaline phosphatase (Fig. 1; Materials and Methods). The *inv-phoA* fusions were then recombined onto the *Y. pseudotuberculosis* chromosome by using a plasmid that was unable to replicate in this organism. By using the two-step strategy described in Fig. 4, we were able to construct two types of strains. One contained a single disrupted copy of *inv* that was fused to *phoA*, while the other was merodiploid and contained an intact copy of *inv* in addition to the fusion. Alkaline phosphatase activity was then assayed to determine whether *inv* expression is temperature regulated and, if so, whether this control is autogenous.

TABLE 3. Temperature-regulated penetration of *Y. pseudotuberculosis* into HEp-2 cells[a]

Strain	Mean % entry at[b]:	
	28°C[c]	37°C[c]
SY327(λ pir)(pRI233)[d]	6.0 ± 2.4	10.1 ± 2.5
SY327(λ pir)(pIPh1)	0.02 ± 0.005	0.01 ± 0.005
YPIII(P⁻)[e]	6.8 ± 3.5	0.4 ± 0.15
YPh110	0.02 ± 0.005	0.04 ± 0.02

[a] Intracellular bacteria were quantitated as described in Materials and Methods. Viable counts were determined on L agar plates after 24 h of incubation at 28°C.

[b] Percentages of total viable counts that survived incubation in the presence of gentamicin represent average of three determinations each.

[c] Temperature at which bacteria were cultured before assay for penetration. Bacteria were grown overnight at 28°C in L broth before 1:100 dilution into the same medium. Cultures were then grown for an additional 4 h at the temperatures indicated before introduction to the cell monolayer.

[d] *E. coli* K-12 strain harboring the *inv* locus (Table 1).

[e] Parental *Y. pseudotuberculosis* strain (Table 1).

TABLE 4. Expression of *inv-lacZ* fusions independently of temperature[a]

Plasmid	Mean β-galactosidase activity at:	
	28°C	37°C
pRI203	0	0
pRI211	67 ± 12	75 ± 15
pRI212	76 ± 16	77 ± 11
pRI203hyb2	43 ± 10	57 ± 9
pRI203hyb5	48 ± 8	51 ± 5
pRI203hyb33	50 ± 11	59 ± 9
pRI203hyb41	57 ± 9	67 ± 12

[a] *E. coli* M8820Mu⁺ containing *inv-lacZ* gene fusions was grown to an A_{550} of 0.5 in L broth containing 100 μg of ampicillin per ml at the temperatures shown. Cells were washed in phosphate-buffered saline and assayed in triplicate for β-galactosidase activity as previously described (13).

Two *E. coli* strains harboring *inv-phoA* fusions and *Y. pseudotuberculosis* derivatives containing the identical constructions were cultured at 28 or 37°C before assay for alkaline phosphatase expression. As with strains harboring the *inv-lacZ* fusions, the *E. coli* strains showed no temperature regulation of *inv* expression (Table 5). This was in marked contrast to the *Y. pseudotuberculosis* derivatives, which showed three- to fourfold depression of *inv-phoA* expression at 37°C. This effect was independent of whether an intact *inv* gene was present in these fusion strains, so the regulation does not appear to be autogenous (Table 5). *Inv* gene expression, therefore, responds to temperature-dependent regulatory factors encoded on the *Y. pseudotuberculosis* chromosome that are not present in *E. coli*, and we conclude that thermoregulation of cell penetration in the parental *Yersinia* organism can be at least partly attributed to thermal control of the *inv* gene.

It could be argued that the hybrid proteins behave anomalously with respect to their stability at the elevated temperature and do not reflect the true behavior of the wild-type *inv* protein. To eliminate this possibility, we analyzed the steady-state levels of native invasin produced by *Y. pseudo-*

TABLE 5. Temperature regulation of *inv-phoA* fusions in *Y. pseudotuberculosis*

Strain (plasmid or fusion)	Mean alkaline phosphatase activity at[a]:	
	28°C	37°C
E. coli		
SM10(pIPh1)	21 ± 3.2	33 ± 4.5
SM10(pIPh7)	36 ± 4.1	49 ± 8.1
SM(pRI233*inv*::*kan*)[b]	0.8	1.2
Y. pseudotuberculosis		
YP202 (*inv*::*kan*)[c]	0.3	0.25
YPh10 (*inv*⁺/*inv-1*::*phoA*)[d]	19.5 ± 2.2	5.5 ± 0.4
YPh110 (*inv-1*::*phoA*)	13.7 ± 1.4	4.0 ± 2.0
YPh70 (*inv*⁺/*inv-7*::*phoA*)	21.2 ± 4.2	7.2 ± 1.4
YPh170 (*inv-7*::*phoA*)	16.5 ± 0.6	5.0 ± 2.1

[a] Strains containing *inv-phoA* fusions were grown to mid-logarithmic phase at the designated temperatures in L broth. Alkaline phosphatase activities were assayed in triplicate as previously described (12). Activity is expressed in units as described in the text.

[b] This *E. coli* strain harbors a plasmid containing a Kan^r drug resistance determinant inserted in the *inv* gene.

[c] This *Y. pseudotuberculosis* strain contains an insertion found in pRI233*inv*::*kan* strain.

[d] Structures of the *inv* region for *Y. pseudotuberculosis inv*::*phoA* fusion strains are shown in Fig. 4.

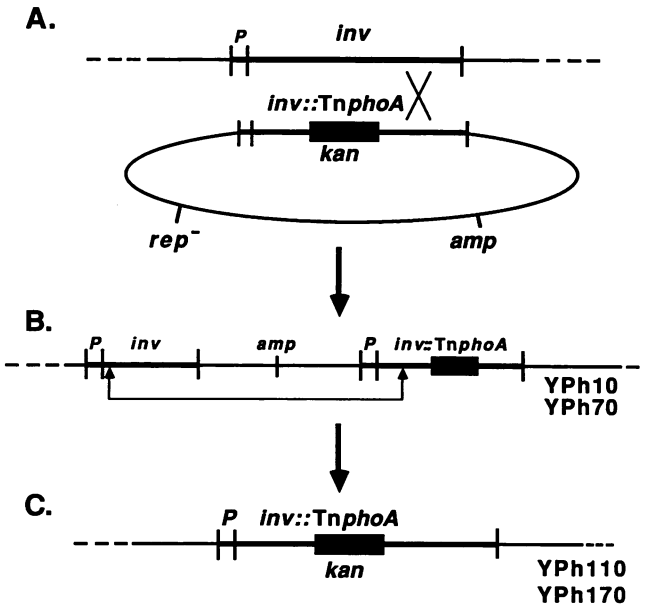

FIG. 4. Isolation of *inv*::Tn*phoA* insertions on the *Y. pseudotuberculosis* chromosome. Tn*phoA* insertion mutations, conferring kanamycin resistance (Kan^r) and resulting in protein hybrids between invasin and alkaline phosphatase, were isolated in the replication-deficient plasmid pRI233 as described in Materials and Methods. The plasmids so isolated (designated pRI233 *inv*::Tn*phoA*) were used to cross the mutations onto the *Y. pseudotuberculosis* chromosome via homologous recombination. (A) Plasmids were mobilized from *E. coli* SM10 (λ *pir*) into *Y. pseudotuberculosis* YPIII(P$^-$) derivatives by selecting for one of the drug resistance determinants (either Amp^r or Kan^r). Stable inheritance of the drug resistance required that the plasmid be integrated into the *Y. pseudotuberculosis* chromosome via the homology provided by the *inv* locus because the plasmid is unable to replicate in this microorganism (9, 13a). (B) Merodiploids, containing both intact and mutated copies of the *inv* gene were then screened for loss of the Amp^r marker that resulted from a second independent crossover (arrows). Numbers at the right denote *Y. pseudotuberculosis* strain numbers that have the merodiploid genetic structure; YPh10 is derived from *inv-1*::Tn*phoA*, while YPh70 is derived from *inv-7*::Tn*phoA*. (C) Segregants that retained the Kan^r phenotype had lost plasmid sequences as well as the intact copy of the *inv* locus. YPh110 and YPh170 are the haploid *inv*::Tn*phoA* strains derived from YPh10 and YPh70, respectively.

tuberculosis at 28 and 37°C. Bacterial membranes were prepared from both *Y. pseudotuberculosis* and an isogenic *inv* mutant strain (Table 1, YP202) grown at these two temperatures. The expression levels of invasin in these extracts were analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotting with a monoclonal antibody that recognizes invasin (gift of John Leong). Invasin was produced in larger amounts when the bacteria were grown at the lower temperature (Fig. 5, compare lanes A and B). The difference in expression observed at 28 and 37°C is consistent with the three- to fourfold difference observed with the alkaline phosphatase assays. As a control, this blot also showed that an *inv* insertion mutant was unable to make significant steady-state antigen levels at either temperature (Fig. 5, lanes C and D). We conclude, therefore, that expression of both the *inv-phoA* fusions and the wild-type

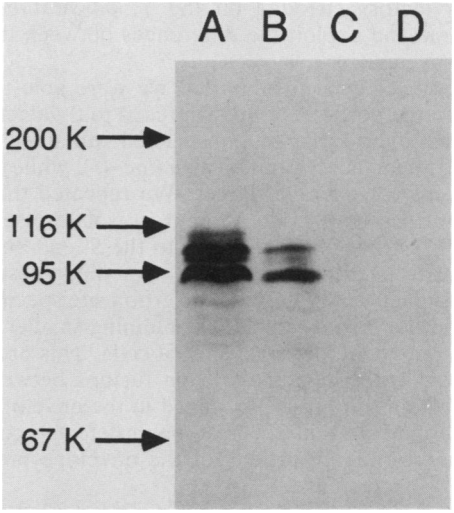

FIG. 5. Temperature regulation of invasin expression in *Y. pseudotuberculosis*. YPIII(P$^-$) and YP202*inv* were grown with aeration in 50 ml of L broth to an A_{600} of 0.8 at either 28 or 37°C. Cells were pelleted, washed in 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 8.0), resuspended in 1.0 ml of the same buffer, and lysed by sonication. Membrane material was washed twice in HEPES buffer and resuspended at a protein concentration of 5 mg/ml. A 20-μl volume of material was then fractionated on 7.5% SDS-polyacrylamide gel, transferred to nitrocellulose, and probed with a monoclonal antibody that recognizes invasin (1B10; gift of John Leong). Lanes: A, YPIII(P$^-$) grown at 28°C; B, YPIII(P$^-$) grown at 37°C; C, YP202*inv* grown at 28°C; D, YP202*inv* grown at 37°C. The numbers on the left indicate molecular weight.

inv gene is depressed by growth at 37°C and that the *inv-phoA* fusion accurately reflects expression of the wild-type *inv* gene.

DISCUSSION

We have shown that gene fusions between the *inv* locus and *lacZ* generated active β-galactosidase hybrids that were translated in the identical direction and fused to the same gene product. Analysis of truncated proteins produced by insertion mutations in this locus was also consistent with the orientation of translation denoted in Fig. 1. That the *inv-lacZ* fusions resulted in hybrid proteins consisting of an invasin amino terminus and a β-galactosidase carboxyl terminus was supported by immunological data. We were also able to purify the product of one of the *inv-lacZ* fusions, raise antiserum, and show that the antibody was able to immunoprecipitate the intact invasin protein (data not shown).

The regulatory properties of the *inv-lacZ* fusion strains emphasize a major difference between *E. coli* and *Y. pseudotuberculosis*. The expression of many gene products, as well as the ability to enter animal cells, is regulated by temperature in the *Yersinia* species (1, 3). We have shown here that the *inv-phoA* fusions crossed onto the *Y. pseudotuberculosis* chromosome are similarly temperature regulated. In contrast, *E. coli* strains encoding *inv* show no such regulatory properties, and neither *inv-lacZ* nor *inv-phoA* fusions were regulated by temperature in this organism. We believe that there must be loci encoded on the *Y. pseudotuberculosis* chromosome that control this temperature response in *trans* that were not present on molecular clones containing the *inv* locus. The strains isolated in the experiments described here should allow us to analyze these

regulatory factors encoded on the *Y. pseudotuberculosis* chromosome and exploit the differences between these two bacterial species.

The only *lacZ* gene fusions that we were able to isolate contained large portions of invasin fused to β-galactosidase. The shortest hybrid that we constructed contained approximately 500 amino acids from invasin (*lac-41*), while the other hybrids were considerably larger. We repeated the experiments described here in an attempt to isolate insertions of Mu d*lac*II1734 that mapped closer to the 5′ end but continued to isolate insertions that were near those described in Fig. 1. Inasmuch as Mu has little insertion site specificity, we believe that the *inv-lacZ* fusions containing smaller portions of invasin may be lethal for bacterial cells. This proposition is consistent with previous data on fusions between cytoplasmic proteins and those localized in the envelope (7, 10, 17), which indicate that severe growth defects occur when small amino-terminal portions of the envelope protein are fused to β-galactosidase.

Our data support and extend our previous results indicating that *inv* is a single gene encoding the protein invasin. The simplicity of this system and the ability to reconstruct the entry process totally by using the well-characterized strain *E. coli* K-12 make this an ideal system for studying cellular penetration by intracellular parasites. In the future, it should be possible to gain considerable insight into the details of this phenomenon by further immunochemical and genetic analysis of invasin.

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