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Specific Detection of *Salmonella typhimurium* Proteins Synthesized Intracellularly

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Studies of the proteins *Salmonella typhimurium* synthesizes under conditions designed to more closely approximate the in vivo environment, i.e., in cell and tissue culture, are not easily interpreted because they have involved chemical inhibition of host cell protein synthesis during infection. The method which we have developed allows specific labeling of bacterial proteins without interfering with host cell metabolic activities by using a labeled lysine precursor which mammalian cells cannot utilize. We have resolved the labeled proteins using two-dimensional electrophoresis and autofluorography. We were able to detect 57 proteins synthesized by *S. typhimurium* during growth within a human intestinal epithelial cell line. Of the 57 proteins detected, 34 appear to be unique to the intracellular environment, i.e., they are not seen during growth of the bacteria in tissue culture medium alone. Current (and future) efforts are directed at organizing the 34 proteins into known stress response groups, determining the cellular locations of the proteins (outer or inner membrane, etc.), and comparing the pattern of proteins synthesized within an intestinal epithelial cell to the pattern synthesized during growth within other tissues.

Members of the genus *Salmonella* are responsible for a diverse variety of illnesses, including typhoid fever, food poisoning, gastroenteritis, and septicemia (31). *Salmonella typhimurium*, the species most frequently isolated from humans (20), also causes a lethal infection in susceptible strains of mice which resembles human typhoid fever (23). The pathogenesis of *Salmonella* infection is a multifactorial process. They are able to attach to eucaryotic cells, invade (15, 17), and survive intracellularly (11) due to the variety of virulence factors which they are capable of producing. These virulence factors include surface antigens, endotoxin, and enterotoxin (13). It is to be expected that *S. typhimurium* would express certain of its virulence factors only when they were needed, i.e., during growth within a suitable host, where study of the microorganism and the proteins it synthesizes is much more difficult than the studies done of in vitro cultures.

Facultative anaerobes such as *Salmonella* respond to the particular constraints of their diverse environments through a continually varying expression and repression of groups of genes, each of which is designed to respond to specific cultural conditions (29, 33–35, 37, 38). This phenomenon is termed "global regulation" (16). In vitro studies have been done which delineate the groups of proteins *Salmonella* synthesizes while under various environmental conditions, and not surprisingly, many of the genes involved in global regulation were also found, upon performance of animal studies, to affect virulence of the organism (27). However, the corresponding studies of *Salmonella* proteins synthesized under conditions approximating the in vivo environment (i.e., in cell and tissue culture), when expression of virulence factors is more likely than during in vitro growth, are not as amenable to interpretation as they have involved the chemical inhibition of host cell protein synthesis during infection (8). Under these conditions *Salmonella* is no longer interacting with a cell which is capable of defense or response, which may result in detection of artifacts or the inability to detect important proteins. In this study we have developed a technique which no longer requires the halting of host cell protein synthesis to specifically label bacterial proteins synthesized intracellularly, resulting in a more relevant "in vivo" model.

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MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used in this study are listed in Table 1. The χ4335 strain was isolated in this lab by Andrew Portteus. The Δ*asd* mutation results in an obligate requirement for diaminopimelic acid (DAP), which is the biosynthetic precursor of lysine as well as a component of the peptidoglycan layer. χ4335 is a low-DAP-requiring strain which is able to grow and survive with only 5 μg of DAP/ml in a rich medium such as Luria broth (LB) (22) as opposed to the 50 μg/ml required by the parent Δ*asd* mutant, χ4288. It requires a higher concentration (10 μg/ml) in more defined media such as tissue culture media. Strains were cultured initially at 37°C with aeration for 18 h in LB containing DL-α,ε-DAP (5 μg/ml) (Sigma, St. Louis, Mo.). Prior to performing invasion assays and intracellular labeling studies, the bacteria were inoculated at a 1:100 dilution into prewarmed (37°C, 5% CO_2) Eagle's Minimal Essential Medium Deficient (MEMD; Gibco BRL, Gaithersburg, Md.) lacking in leucine, methionine, and lysine and supplemented with 0.2% glucose and DAP (10 μg/ml). Additional supplements included L-histidine HCl (10 μg/ml), DL-methionine (20 μg/ml), and DL-threonine (80 μg/ml). Cultures were then incubated (nonaerated) in a 5% CO_2 atmosphere for 4 h at 37°C before use. Fetal calf serum (FCS) was not present in MEMD when used for culturing bacteria or performing invasion assays. Studies involving the incorporation of *meso*-[3,4,5-^{3}H]DAP ([^{3}H]DAP; American Radiolabeled Chemicals, Inc., St. Louis, Mo.) into χ4335 alone were performed in MEMD supplemented with 10 μg of DAP/ml.

Cell culture conditions. Human embryonic intestinal epithelial cell line Intestine 407 (Int-407, formerly called Henle-407; purchased as ATCC CCL6 from the American Type Culture Collection, Rockville, Md. [19]) was maintained in Eagle's minimal essential medium (EMEM) containing 10% FCS and 2 mM glutamine. Working stocks were passaged every 3 to 5 days for up to 15 passages. Cells were seeded into either 24-well plates (10^5 cells/ml/well) or at 5×10^5 cells/ml in 25-cm^2 tissue culture flasks (Costar, Cambridge, Mass.) for invasion assays and intracellular labeling studies, respectively.

Incorporation of [^{3}H]DAP by χ4335 and χ3339. The abilities of χ4335 and its wild-type parent, χ3339, to incorporate [^{3}H]DAP were determined as follows. The strains were inoculated at a 1:100 dilution into MEMD containing DAP (10 μg/ml) and grown with aeration for ~2 h (average cell number, 1.4×10^7). [^{3}H]DAP was added at a concentration of 10 μCi/ml, and 50-μl samples were taken at intervals ranging from 15 to 30 min. Samples were spotted on Whatman no. 3 filters (Whatman International, Ltd., Maidstone, England), allowed to air dry, placed into ice-cold 10% (wt/vol) trichloroacetic acid (TCA), washed twice with cold 5% TCA and twice with cold 95% ethanol, and again allowed to air dry. The filters were placed in 1.5 ml of Aquamix scintillation fluid (ICN Radiochemicals, Irvine, Calif.) and counted on a 1410 Liquid Scintillation Counter (LSC;

TABLE 1. *S. typhimurium* and *E. coli* strains

Strain	Genotype and relevant characteristics	Reference or source
χ3000	*S. typhimurium* LT2-Z prototroph, contains 91 kb of pStLT100	18
χ3021	*S. typhimurium* LT2-Z Δ*asdA1* $P22^i$	This lab
χ3339	*S. typhimurium* SL1344 *hisG xyl* colicin$^+$	18
χ3447	F^- *hsdL6* Δ[*gal-uvrB*] *flaA66 rpsL120 xyl-404 lamB*$^+$ (*E. coli*) Δ[*zja*::Tn*10*] *hsdSA29*	25
χ3520	Obtained by transduction of χ3000 with P22 HT*int* (χ3536) constructed with *zhf-4*::Tn*10*, Δ*asdA1 zhf-4*::Tn*10*	This lab
χ3536	Selected as an Asd$^+$ Tc^r derivative from a transduction of χ3021 with P22 HT*int* grown on a Tn*10* library in χ3000 *zhf*::Tn*10*	This lab
χ3918	*S. typhimurium* LT2 pStLT203$^+$ *hsdL6* Δ(*gal-uvrB*)*1005 flaA66 rpsL120 xyl-404 lamB*$^+$ (*E. coli*) Δ(*zja*::Tn*10*) *hsdSA29 val*	36
χ4240	χ3339 transduced with P22 HT*int* (χ3520) carrying Δ*asd*, cotransduces with *zhf-4*::Tn*10*, *hisG rpsL xyl* Δ*asdA1 zhf-4*::Tn*10* colicin$^+$	A. Portteus
χ4241	χ4240 selected for fusaric acid resistance, Tc^s, *hisG rpsL xyl* Δ*asdA1* Δ[*zhf-4*::Tn*10*] colicin$^+$	A. Portteus
χ4288	χ4241 transduced with P22 HT*int* (χ3918) and selected for Tc^r encoded by Tn mini-*tet* on pStSL101*hisG rpsL xyl* Δ*asdA1* Δ[*zhf-4*::Tn*10*], colicin$^+$	A. Portteus
χ4335	Selected as a derivative of χ4288 able to grow on low concentrations of DAP (5 μg/ml), *hisG rpsL xyl* Δ*asdA1* Δ[*zhf-4*::Tn*10*]	A. Portteus
HB101	*E. coli* K-12 x *E. coli* B hybrid, *glnV44 hsdS20*(r_B^- m_B^-) *recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1*	7

Wallac, Gaithersburg, Md.). Results were expressed as kilocounts per minute per milliliter.

Isotopic labeling of bacterial strains. The incorporation of [^{3}H]DAP into the protein and peptidoglycan fractions of χ4335 was determined as described by Wientjes et al. (39) with some modification. χ4335 was inoculated into MEMD at a 1:100 dilution and grown at 37°C with aeration for ~2 h (average cell number, 1.4×10^7). [^{3}H]DAP was added at a concentration of 10 μCi/ml, and sampling began at this time. At 30- to 60-min intervals, 4 ml of culture was removed, and duplicate 1-ml samples were filtered through Millipore HAWP 0.45-μm-pore-size filters (Oxford Glycosystems [formerly Millipore Corp.], Bedford, Mass.). These were the whole-cell counts. The filters were allowed to air dry and then placed in Aquamix scintillation fluid for counting. Two samples of 50 μl each were spotted onto Whatman no. 3 filters and treated as described above. These were the duplicate protein counts. Two samples of 1 ml each were mixed 1:1 with boiling 10% (wt/vol) sodium dodecyl sulfate (SDS; Oxford Glycosystems) and boiled for 30 min. The samples were then filtered over Millipore HAWP 0.45-μm-pore-size filters which had been presoaked in cold DAP (10 mg/ml), washed five times with 10 ml of boiling H_2O, allowed to air dry, and placed in Aquamix scintillation fluid. These were the duplicate peptidoglycan counts. All filters were counted in the LSC. Protein and peptidoglycan percentages were calculated based on whole-cell counts.

Uptake and incorporation of [^{3}H]DAP into Int-407 cells. Int-407 cells were seeded into 6-well plates (Costar) and grown as described above to 90% confluency. MEMD supplemented with [^{3}H]DAP (10 μCi/ml) was added and monitored for both the uptake of the label and its incorporation into protein. Uptake was measured by harvesting the whole cells at 3 h with a PHD Cell Harvester (Cambridge Technology, Inc., Watertown, Mass.). Filters were placed in TS2 Tissue Solubilizer (RPI, Mount Prospect, Ill.) and incubated at 50°C for 2 h, cooled to room temperature, mixed with 6 ml of Aqualume Scintillation cocktail (ICN Radiochemicals), and analyzed by two separate methods. Monolayers were lysed with 0.1% sodium deoxycholate (Na-DOC; Sigma) in phosphate-buffered saline (PBS, pH 7.2), and samples were precipitated with TCA and counted in the LSC. Acetone-precipitated Int-407 proteins were analyzed by two-dimensional (2-D) electrophoresis and autofluorography (described below).

Invasion assays. The effect of the DAP concentration required by χ4335 on its ability to invade at levels comparable to its wild-type parent, χ3339, was determined by performing invasion assays with Int-407 in the presence or absence of cold DAP (10 μg/ml). Int-407 cells were seeded at 1×10^5 to 2×10^5 per well in 24-well plates and were used at 90% confluency. One set of plates was incubated with DAP (10 μg/ml) and one was incubated without DAP. χ4335 and χ3339 were inoculated at 1:100 dilutions from standing overnight cultures into fresh, prewarmed MEMD containing DAP (10 μg/ml) and incubated in a 5% CO_2 atmosphere at 37°C. Cultures were grown for 4 h, spun down at 2,200 × *g* and resuspended in fresh prewarmed medium either in the presence or absence of DAP. Wells were washed two times with Hank's balanced salts solution (HBSS), and the bacterial suspensions were added at 1 ml per well (multiplicity of infection of approximately 10:1). Each strain was tested in triplicate wells in three separate experiments. Assays were performed in the presence or absence of DAP. *Escherichia coli* HB101 served as the noninvasive control. Bacteria were allowed to attach and invade in a 5% CO_2 atmosphere for 2 h at 37°C, and the monolayers were then washed three times with HBSS. Fresh MEMD containing gentamicin (100 μg/ml) and DAP (10 μg/ml) or MEMD containing gentamicin only was placed on the monolayers, and they were further incubated for 2 h. Wells were washed three times with PBS and lysed with 0.1% Na-DOC to release intracellular bacteria. Serial dilutions were plated on LB plates containing DAP (5 μg/ml) and incubated at 37°C. CFU were enumerated the following day, and the percent invasion was calculated as a percentage of the total inoculum.

Intracellular labeling protocol. Int-407 cells were seeded at 5×10^5 cells/ml and allowed to grow to near 80% confluency in 25-cm^2 tissue culture flasks. They were then incubated for 16 to 18 h in MEMD containing [^{3}H]DAP (10 μCi/ml). χ4335 was prepared by inoculating a 1:100 dilution from an aerated overnight LB culture into 20 ml of prewarmed MEMD supplemented with amino acids, glucose, and cold DAP (10 μg/ml) and containing no FCS. The culture was incubated in a 5% CO_2 atmosphere for 4 h at 37°C and then spun down at 2,200 × *g* for 10 min. The pellet was resuspended in 3 ml of the same medium containing [^{3}H]DAP (50 μCi/ml). Monolayers were washed two times with prewarmed HBSS to remove all FCS and then inoculated with the χ4335 suspension. All incubations were done in a 5% CO_2 atmosphere at 37°C. Attachment and invasion was allowed to proceed for 2 h. Supernatant fluid was then removed, the monolayers were washed three times with HBSS and fresh MEMD containing gentamicin (100 μg/ml), and [^{3}H]DAP (50 μCi/ml) was added. The kill step continued for 2 h, and the monolayers were then washed three times with PBS and lysed with 0.5 ml of boiling Sample Buffer I (0.3% [wt/vol] SDS, 200 mM dithiothreitol, 28 mM Tris-HCl, 22 mM Tris base), boiled further for 5 min, and then placed on ice for 5 min. Sample Buffer II (24 mM Tris base, 476 mM Tris HCl, 50 mM $MgCl_2$, DNase [1 mg/ml], RNase A [0.25 mg/ml]) was added, and the sample was further chilled for 10 min. Acetone (80%) was then added to 80% volume, and the tube was chilled on ice for 20 min. Samples were then spun down at 8,800 × *g* for 10 min, the supernatant was removed, and pellets were allowed to air dry for 5 min. The pellets were resuspended in 50 μl of a 1:4 mixture of Sample Buffer I to Sample Buffer III (9.9 M urea, 4% Nonidet P-40, 2.2% preblended ampholytes [pH 3 to 10, reinforced in the 5 to 7 range], 100 mM dithiothreitol) and analyzed by 2-D electrophoresis (see below). Duplicate samples were run so that one gel could be silver stained and one dried for autoradiography. In order to compare the DAP-labeling protocol to results obtained with ^{35}S labeling in the presence of cycloheximide, we carried out the following procedure. The bacterial culture was grown and subcultured as described above. After spinning the culture down, the pellet was resuspended as above and used to inoculate the Int-407 monolayers. The monolayers had previously been incubated with cold DAP to ensure the survival of χ4335. The bacteria were allowed to attach and invade for 2 h in a 5% CO_2 atmosphere at 37°C. The monolayers were then washed twice with HBSS, and fresh MEMD containing gentamicin (100 μg/ml) was added. Cycloheximide (200 μg/ml) was added followed by Tran^{35}S-label (150 μCi/ml) (ICN Radiochemicals). Tran^{35}S-label contains primarily L-[^{35}S]methionine (70%) and L-[^{35}S]cysteine (15%). Labeling was continued for 2 h. The monolayers were then washed three times with PBS and lysed with a solution of 0.1% Na-DOC in PBS, and the bacterial pellet was spun down at 8,800 × *g*. The pellet was boiled for 5 min in 40 μl of Sample Buffer I, treated with 4 μl of Sample Buffer II, chilled on ice for 10 min, and treated with 160 μl of Sample Buffer III. These preparations were stored at −70°C until used for 2-D electrophoresis.

2-D electrophoresis and autofluorography. 2-D electrophoresis was essentially done by the method of O'Farrell (30) with some modifications. (The equipment used was the Millipore 2-D Investigator, and all reagents were also from Oxford Glycosystems). Protein samples were loaded onto 4.1% acrylamide tube gels for isoelectric focusing in the first dimension. The ampholytes used were a preblended mixture of pH 3 to 10 which was reinforced in the pH 5 to 7 range. Isoelectric focusing was carried out for 18,000 V · h. The second dimension consisted of a large format (22 by 22 cm) 11.5% polyacrylamide slab gel (Duracryl) which was run for 6 h at a maximum voltage of 500. Gels were fixed in a

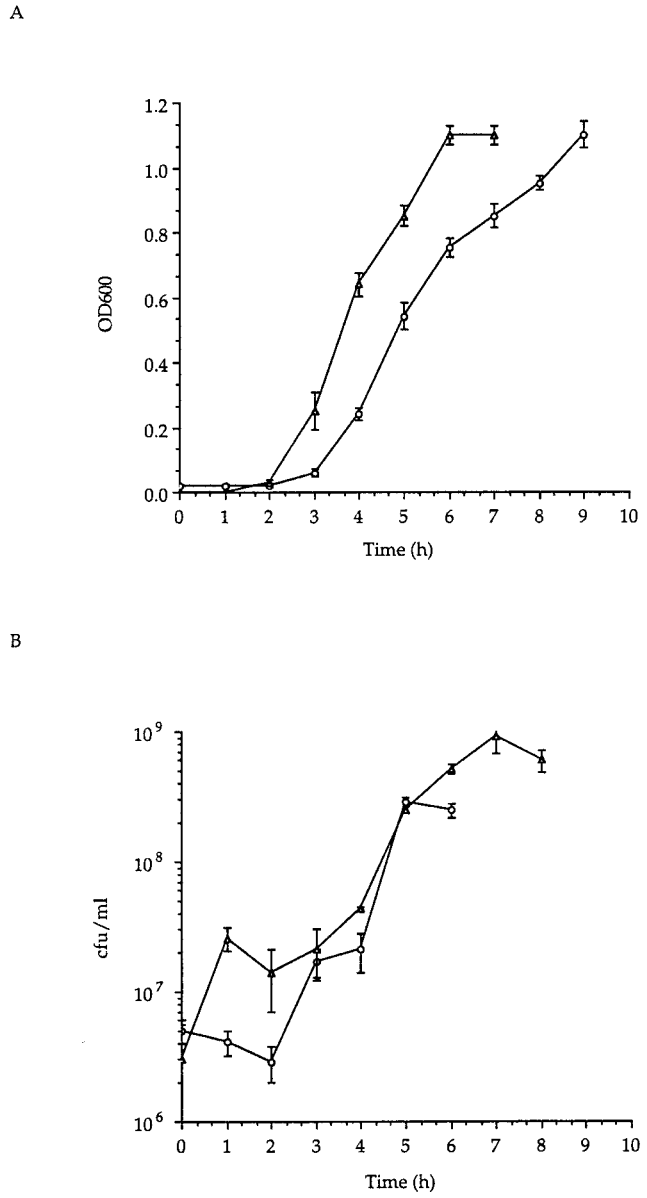

FIG. 1. Growth curve comparisons of χ4335 and χ3339. (A) χ4335 (○) and χ3339 (△) grown with aeration in LB containing 5 μg of DAP/ml. (B) χ4335 and χ3339 grown at 37°C with aeration in MEMD containing 10 μg of DAP/ml. Error bars are shown.

solution of isopropanol-water-acetic acid (65:25:10) for 30 min and then soaked in Amplify (Amersham International, Arlington Heights, Ill.), dried, and exposed to preflashed X-Omat AR X-ray film (Eastman Kodak Co., Rochester, N.Y.) for 4 to 6 weeks for [^{3}H]DAP-labeled proteins or 5 days for Tran^{35}S-labeled proteins. Silver staining was done by the modified Morrisey technique (28) with silver nitrate (Sigma).

Image acquisition and analysis. Gels were scanned into a Sun workstation by using a high-resolution (1,024 × 1,024 pixel) Kodak Microvision CCD camera. Images were analyzed with the BioImage Visage 2-D and Automatic Gel Matcher software. Protein spots are automatically quantified with 36 inflection points per spot and a local background to ensure accuracy and reproducibility among gel data sets. The software uses actual spot boundaries instead of assuming elliptical or Gaussian models of fitting. Quantification includes spot height, width, location, size (mm^2), optical density (OD), integrated intensity (OD/mm^2), and percent integrated intensity. Protein patterns among gels are compared by using the Automatic Gel Matcher software, which employs pattern recognition algorithms and constellation matching instead of *x* and *y* coordinate matching. The standards software assigns molecular weight and pI as defined by the user. Images can be enhanced to improve clarity.

RATIO and NORMALIZE programs are provided by the BioImage Comparator software. RATIO calculates the quantitative ratio of the integrated intensities of the spots contained in two compared images. The ratio is computed by first determining which spots in the image match and then calculating the central tendency of the distribution of the log ratios of the integrated intensities for the matched spots. RATIO corrects for the unavoidable quantitative differences resulting from sample preparation and loading and normalizes the images in the third (*z*) dimension. The NORMALIZE program applies the multiplicative factor obtained from RATIO to the quantitative numbers in the list of all the spots for each gel.

RESULTS

Growth and invasion characteristics of χ4335. χ4335 was chosen for this study because the obligate requirement for DAP ensured uptake of the label, and the fact that it required lower levels of DAP to survive gave it an advantage over the parent Δ*asd* mutant (χ4288) in the intracellular environment where sustaining high levels of DAP would be difficult and costly. Growth curves done in a rich medium such as LB revealed that χ4335 could attain cell densities comparable to those of wild-type χ3339 when DAP concentrations were as low as 5 μg/ml (Fig. 1A). However, in defined media such as MOPS (morpholinepropanesulfonic acid) (data not shown) and MEMD (Fig. 1B) somewhat higher DAP concentrations were required to get adequate growth of χ4335. In addition, it was observed that the uptake kinetics differed with various media. The maximum uptake in MOPS was attained at ~2 h (data not shown), while uptake continued to increase past the 2-h time point during growth in MEMD. Since higher concentrations of cold DAP naturally resulted in lower incorporation of label, we chose a final DAP concentration of 10 μg/ml in MEMD to obtain adequate growth of χ4335 and still allow sufficient incorporation (82%) of the label into the protein fraction of the mutant. Invasion assays were therefore performed using a DAP concentration of 10 μg/ml, and the results are shown in Table 2. These data show that χ4335 can grow and invade at near wild-type levels when DAP is provided at the required concentration.

Uptake of [^{3}H]DAP by χ4335 and χ3339. In vitro uptake experiments were performed to determine whether χ4335 incorporated [^{3}H]DAP more efficiently than did the wild-type parent, χ3339. The incorporation pattern of the mutant χ4335 was further analyzed to determine the percentage of label found in both the protein and peptidoglycan fractions. DAP is an essential component of the peptidoglycan layer (32) as well as the biosynthetic precursor of lysine (9, 10). It was therefore necessary to determine the amount of label that was lost to the peptidoglycan fraction compared to the amount that eventually localized in the protein fraction. Figure 2 shows the comparison of total [^{3}H]DAP uptake by the mutant and wild-type parent as determined by analysis of TCA-precipitated samples. The results demonstrate that χ4335 incorporates the label much more quickly during the early time points. While χ3339 eventually reaches a comparable level of incorporation it does

TABLE 2. Invasion of Int-407 in the presence and absence of DAP

Strain	Percent invasion[a]	
	0 μg/ml DAP	10 μg/ml DAP
χ3339	72 ± 17	89 ± 35
χ4335	14 ± 12	72 ± 14
HB101	<0.01	<0.01

[a] Percent invasion was calculated as the percentage of the total inoculum added. Data are the means ± standard errors from triplicate wells per strain for three separate assays.

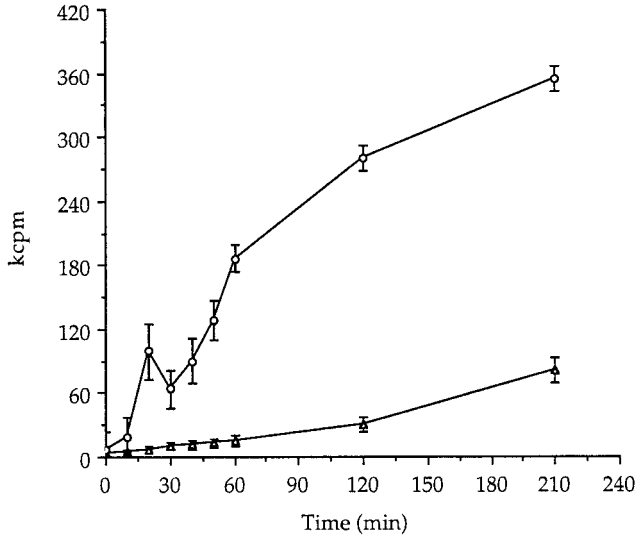

FIG. 2. Comparison of the uptake of [^{3}H]DAP by χ4335 (○) and χ3339 (△) as determined by cpm in TCA-precipitated samples of the cultures grown in MEMD. Sample size was 50 μl, and values shown represent a calculation of counts per milliliter. Error bars are shown.

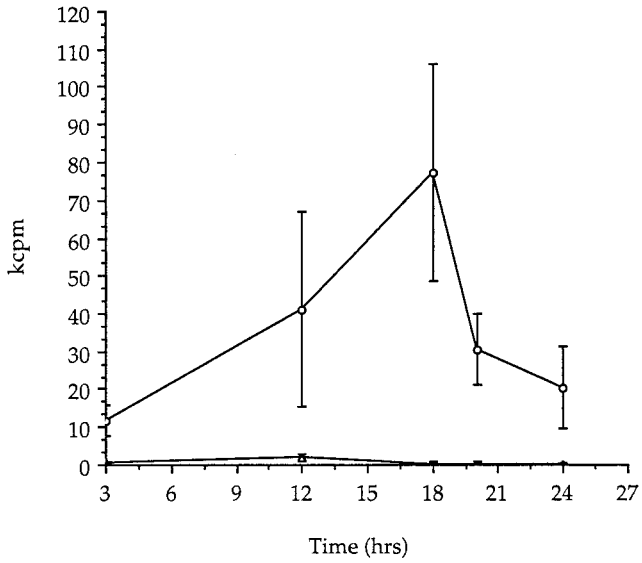

FIG. 4. Uptake of [^{3}H]DAP by Int-407 cells (○) determined by harvesting whole cells and analyzing the counts present, and analysis of TCA-precipitated samples of the monolayers (△).

so much later, past the standard 4-h time course allowed for the invasion studies. Addition of higher concentrations of cold DAP (50 μg/ml) resulted in lower incorporation of label, as expected (data not shown). As χ4335 was observed to take up a greater amount of label during the required time range, we further analyzed the pattern of incorporation to determine the percentage of label present in the protein fraction. Since TCA treatment results in precipitation of the peptidoglycan as well as the protein fractions, we utilized the method of Wientjes et al. (39) to separate these two fractions and determine if the level of incorporation into protein was sufficient for this study. The peptidoglycan fraction was separated by boiling in SDS as described above. Figure 3 shows the incorporation of [^{3}H]DAP into the peptidoglycan (SDS insoluble) and protein fractions of χ4335. It was observed that approximately 82% of the label was present in the protein fraction over a 4-h time span, and uptake continued to increase, in contrast to the pattern seen during growth in MOPS.

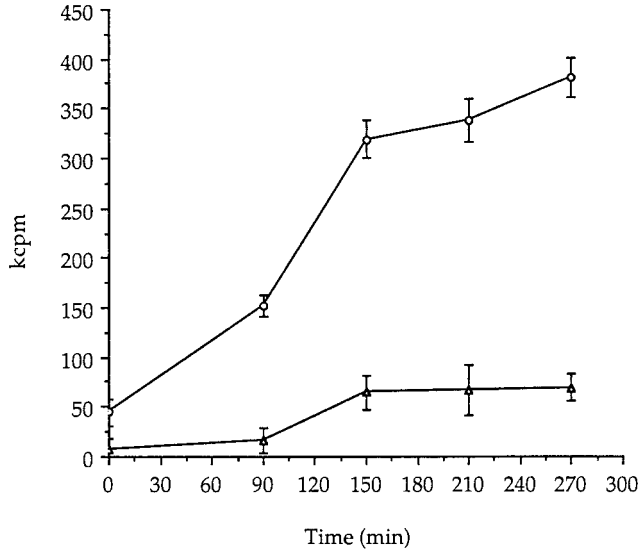

FIG. 3. Incorporation of [^{3}H]DAP into the protein (○) and peptidoglycan (△) fractions of χ4335 grown in MEMD. Sample size for protein determination was 50 μl, and values shown represent a calculation of counts per milliliter. Sample size for peptidoglycan determination was 1 ml. Error bars are shown.

Uptake of [^{3}H]DAP by Int-407. In order for the intracellular labeling scheme to work it was necessary that the host cell be permeable to the label but not use the label for incorporation into its own proteins. Mammalian cells do not utilize DAP as a precursor to lysine (21). This essential amino acid cannot be synthesized but must be environmentally provided (6). It was possible, however, that the Int-407 cells might degrade DAP, thereby freeing the label for use in other incorporation pathways. In order to test these possibilities the Int-407 cells were incubated in the presence of [^{3}H]DAP for a variety of time points to determine if the cells accumulated it and whether their proteins became labeled. The data given in Fig. 4 indicate that Int-407 cells are permeable to DAP and that intracellular levels of the label reach maximum levels at approximately 18 h. Between 18 and 20 h the amount of intracellular label began to decline. Int-407 cellular protein was monitored for possible incorporation by counting TCA-precipitable samples and by running samples of acetone-precipitated protein on 2-D gels and analyzing them by autofluorography. Figure 4 also shows that the amount of TCA-precipitable counts obtained from lysed Int-407 monolayers was negligible. Figure 5 shows the results of autofluorography. Figure 5A represents the silver-stained 2-D gel of the acetone-precipitated cellular proteins of an Int-407 monolayer harvested between 18 and 20 h after addition of the label. The gel is oriented with the basic side at the left and the acidic side at the right. Figure 5B represents the autofluorogram of a duplicate gel after a 4-week exposure. It can be seen that Int-407 cells do not incorporate [^{3}H]DAP into their cellular protein, at least not at levels that can be detected by autofluorography.

Intracellular labeling of χ4335 proteins. Figure 6B shows a silver-stained gel of the proteins synthesized by χ4335 during growth in MEMD. Figure 6A represents an autofluorogram of the proteins synthesized by χ4335 during growth in MEMD.

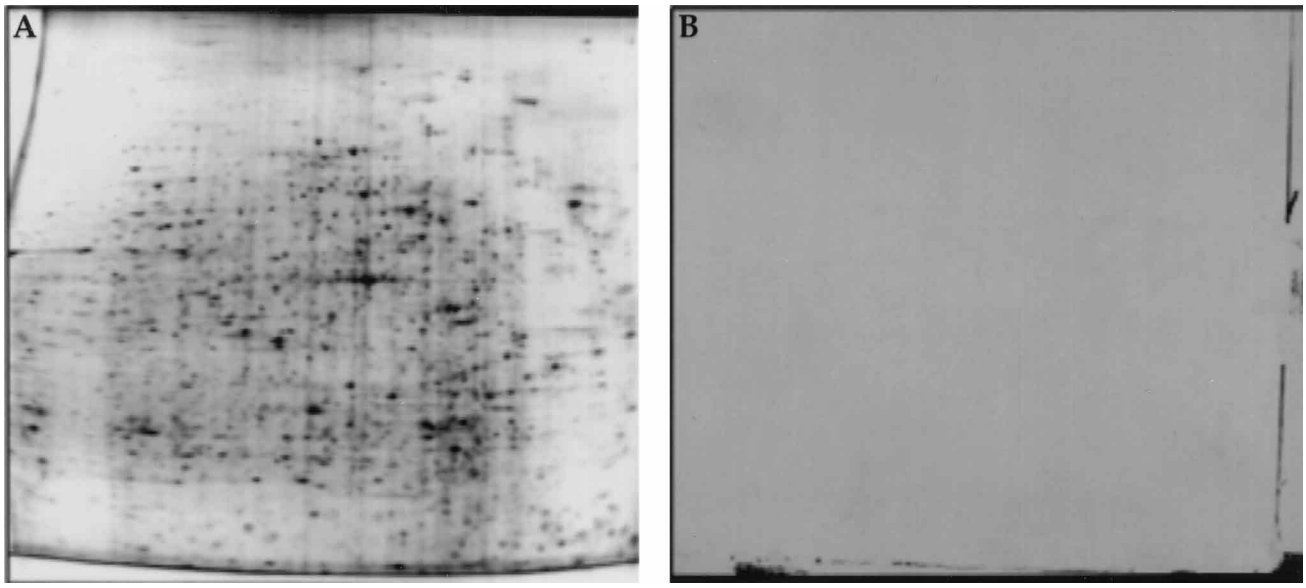

FIG. 5. 2-D SDS-PAGE and autofluorography of Int-407 whole-cell proteins incubated in the presence of [^{3}H]DAP. (A) Silver-stained 11.5% polyacrylamide gel of Int-407 whole-cell proteins. Gel is oriented from left to right as basic to acidic. The pH range of the ampholytes is 3 to 10 and was reinforced in the 5 to 7 range. (B) Autoradiogram of a duplicate sample of the gel shown in panel A. The dried gel was exposed to X-Omat AR Kodak film at −70°C for 4 weeks.

The pattern of proteins synthesized intracellularly by χ4335 is shown in Fig. 7. Figure 7A represents the silver-stained 2-D gel of the cellular proteins of both Int-407 and χ4335 as obtained from an infected monolayer 4 h after inoculation. Figure 7B represents the autofluorogram of a duplicate sample after a 4-week exposure. In this experiment the entire monolayer was added, whereas in subsequent experiments we separated the bacterial and host cells after 4 h to increase the proportion of bacterial protein which can be loaded onto the gel. The gel and autofluorogram were scanned and analyzed using the software described above. When using the [^{3}H]DAP label, we found 57 proteins that appear to be synthesized within the epithelial cell. Comparison to the pattern of proteins synthesized by χ4335 in tissue culture medium alone (Fig. 6B) demonstrated that 34 of these 57 proteins appear to be unique to the process of infection of the epithelial cell (Table 3).

In order to observe the reaction of χ4335 to an inert host cell we followed the standard procedure of inhibiting host cell protein synthesis with cycloheximide while labeling the bacterial protein with Tran^{35}S-label (1). The higher specific activity of the Tran^{35}S-label enabled us to detect 146 proteins synthesized intracellularly by χ4335. We compared the 34 unique intracellular [^{3}H]DAP-labeled proteins described above to this group and found that 9 of the 34 were synthesized in both the

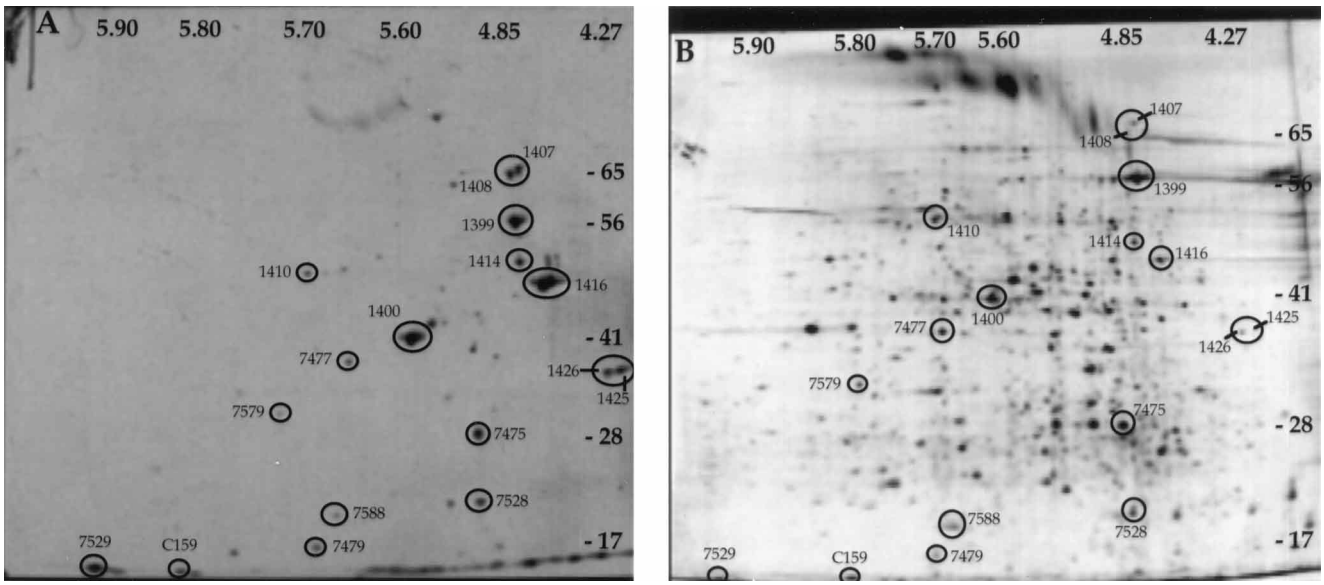

FIG. 6. Whole-cell lysate proteins of χ4335 grown in MEMD and labeled with [^{3}H]DAP for 4 h. Identical proteins are circled in each panel. (A) Autoradiogram of a duplicate sample of the gel shown in panel B. The dried gel was exposed to X-Omat AR Kodak film at −70°C for 4 weeks. (B) Silver-stained 11.5% polyacrylamide gel of χ4335 whole-cell proteins. Gel is oriented from left to right as basic to acidic. The pH range of the ampholytes is 3 to 10 and was reinforced in the 5 to 7 range.

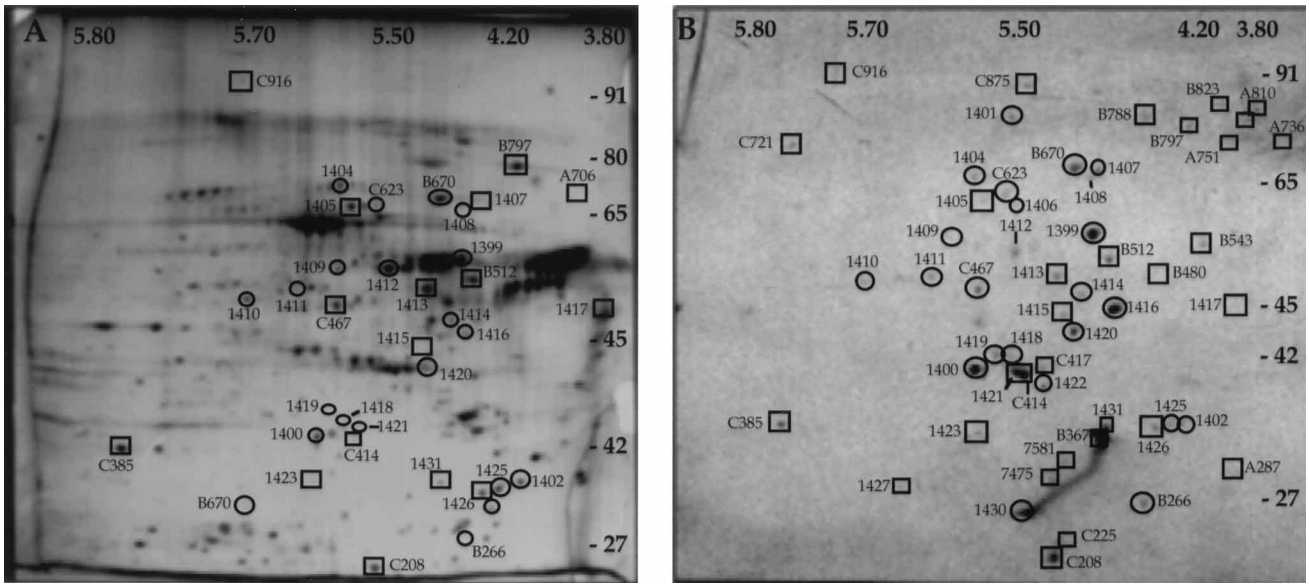

FIG. 7. 2-D SDS-PAGE and autofluorography of whole-cell lysates of Int-407 cells 4 h after infection with χ4335 in the presence of [^{3}H]DAP. (A) Silver-stained gel showing complete cellular proteins. The gel is oriented from left to right as basic to acidic. (B) Autoradiogram of a duplicate sample of the gel shown in panel A. The dried gel was exposed to X-Omat AR Kodak film at −70°C for 4 weeks. Circles represent proteins which are also seen when χ4335 is grown alone in MEMD. Squares indicate representative proteins which are seen only when χ4335 is grown within Int-407 cells.

presence and absence of cycloheximide (Table 3), whereas the remaining 25 were apparently synthesized only in the absence of cycloheximide when the host cell was fully responsive (Table 3).

Figure 8 is a comparison of the protein patterns exhibited by χ4335 during growth within a fully active host cell (Fig. 8A [same as Fig. 7B]) and when host cell protein synthesis has been inhibited by cycloheximide (Fig. 8B). The proteins shown in Fig. 8A are labeled with [^{3}H]DAP, while those shown in Fig. 8B are labeled with Tran^{35}S-label. Proteins which were synthesized under both conditions and proteins which were synthesized intracellularly only when the host cell was fully active are evident.

Some of these intracellularly synthesized proteins are undoubtedly members of various stress-response groups and are induced simply in response to the intracellular environment. Experiments are under way to determine which of these may be thus classified and which are unique proteins which have not yet been described.

DISCUSSION

A number of studies have been done of the genes which are expressed and the proteins which are synthesized during growth within a variety of host cells, including human and murine macrophages (1, 5, 8), epithelial cells (11, 12), and the animal host itself (24). The majority of the studies done with tissue culture cells have involved the use of chemical cessation of host cell protein synthesis in order to allow the specific labeling of bacterial proteins only. In this paper we describe a method of labeling bacterial proteins without shutting down the host cell, so that the effect of a fully interactive environment remains intact.

We chose a Δ*asd* mutant of the pathogenic SL1344 (χ3339) strain of *S. typhimurium*, because this mutation results in an obligate requirement for the biosynthetic lysine precursor DAP. In vitro-labeling studies revealed that this mutant, χ4288, was able to take up [^{3}H]DAP much more quickly than the parent and to incorporate approximately 82% of the label into its protein fraction. When DAP is provided at the required levels, the invasive ability of the mutant is comparable to that of the wild type. The cell line used in this study was Int-407, a human embryonic epithelial cell line. Incubation of these cells with [^{3}H]DAP did not result in incorporation of label into cellular protein, as determined by 2-D SDS-polyacrylamide gel electrophoresis (PAGE) analysis (Fig. 5), although the mammalian cells were permeable to DAP and accumulated sufficient intracellular quantities. It should be noted that the amount and kinetics of [^{3}H]DAP uptake and/or diffusion into mammalian cells can vary significantly among different cell lines and types. In addition, there is some variation to be expected during different trials within the same cell line. It is necessary to perform sufficient preliminary studies with the cell line to be used in order to determine the optimum conditions for maximum uptake. To enhance the intracellular labeling of the bacteria we used a defined tissue culture medium, MEMD, lacking leucine, methionine, and lysine. Leucine and methionine were added to the medium but lysine was left out for all invasion time points, as was FCS, a potential source of amino acids. We had previously determined that the lack of lysine and FCS for the 4-h duration of the labeling experiment had no adverse effects on Int-407 as determined microscopically and with respect to invasion by *Salmonella* (data not shown). It is possible that Int-407 might utilize labeled lysine which had been synthesized by intracellular bacteria and then released, but we believe the short time period precludes this possibility from making a significant impact.

In order to determine the effect of shutting down host protein synthesis on the protein pattern exhibited by χ4335 we compared the protein pattern of [^{3}H]DAP-labeled cells to that of cells labeled with Tran^{35}S-label in the presence of cycloheximide. When using the DAP protocol we found approximately 57 proteins that were synthesized within the Int-407 cells, 34 of which appear to be unique (i.e., they were not seen in χ4335 growing in MEMD alone). The Tran^{35}S-labeling protocol allowed the detection of 146 proteins synthesized during growth

TABLE 3. Proteins synthesized under various intra- and extracellular conditions

Protein synthesized	pI	MW[c]
Intracellular[a]		
Absence of cycloheximide[b]		
A810	3.78	81.0
A782	3.82	78.2
A751	3.98	75.1
A736	3.59	73.6
A287	3.95	28.7
B823	4.02	82.3
B797	4.24	79.7
B788	4.49	78.8
B706	4.08	70.6
B670	4.94	67.0
B543	4.13	54.3
B512	4.73	51.2
B480	4.51	48.0
B367	4.78	36.7
B266[d]	4.50	26.6
C916	5.72	91.6
C875	5.38	87.5
C721	5.76	72.1
C623[d]	5.58	62.3
C467	5.60	46.7
C417	5.21	41.7
C414	5.41	41.4
C385	5.77	38.5
C225	5.04	22.5
C208	5.07	20.8
Presence[e] and absence of cycloheximide		
1405	5.59	61.4
1409	5.60	53.6
1413	5.06	48.4
1415	5.02	45.0
1417	3.84	45.2
1421	5.51	41.5
1423	5.59	35.6
1430	4.70	25.6
1431	4.75	38.0
Intracellular and in vitro[f] growth		
1399	4.84	56.1
1402	4.27	38.6
1407	4.80	66.4
1408	4.85	65.2
1414	4.90	46.4
1416	4.70	45.4
1420	4.95	44.0
7575[d]	4.70	41.4
1425	4.34	38.6
1426	4.44	37.9
1400	5.60	41.7
1401	5.56	79.0
1404	5.60	65.3
1406	5.51	60.8
1410	5.70	47.1
1411	5.64	48.4
1412	5.52	52.5
1418	5.57	42.6
1419	5.58	42.6
1422	5.20	40.7
7581[d]	5.00	32.6
7475[d]	5.11	29.3
1427	5.65	28.7

[a] Bacteria isolated from within Int-407 cells.
[b] No cycloheximide was added and the cells were labeled with [^{3}H]DAP.
[c] MW, molecular weight (in thousands).
[d] Detectable only with computer assistance.
[e] Cycloheximide was added and the cells were labeled with Tran^{35}S-label.
[f] In vitro cultures were grown in MEMD.

within the inert intestinal epithelial cells. The increased number of proteins detected is attributed to the higher specific activity of the ^{35}S label. Upon comparison of the two patterns it was observed that of the 34 [^{3}H]DAP-labeled proteins, 9 were also synthesized in the presence of cycloheximide. However, the most significant observation was the group of 25 proteins synthesized intracellularly by χ4335 only in the absence of cycloheximide, i.e., only when the host cell is active. This indicates that a host cell capable of interaction elicits an entirely different response from χ4335. While it is possible that some proteins may be lacking sufficient cysteine and methionine residues to incorporate the ^{35}S label, we consider it an extremely remote possibility that this would be the case for all of the 25 proteins observed here. This consideration was further strengthened by comparison of the protein patterns exhibited by χ4335 during growth in MEMD with the two different labels ([^{3}H]DAP and Tran^{35}S-label). Although only 103 proteins were detected by using the tritium label, 96 were seen to match with the proteins detected by Tran^{35}S-label (data not shown). This indicates that [^{3}H]DAP and Tran^{35}S-label are not specifically labeling different subsets of proteins. Due to the difficulties inherent in the detection of tritium it would be preferable to use a more readily detectable isotope which could allow the detection of a larger number of proteins within a shorter time period. A ^{14}C-labeled DAP compound would significantly enhance the sensitivity of this technique; however, the synthesis of such a compound, in which only the internal carbons are labeled, is a difficult and expensive procedure.

It should be further noted that, when using the intracellular DAP protocol, the label is present throughout the entire 4-h procedure, whereas when using Tran^{35}S-label, the label is added at the same time as gentamicin. This was done to increase the specific activity of the tritiated proteins as this label is much more difficult to detect than ^{35}S. We recognize this discrepancy and are aware of the possibility that some proteins detected by [^{3}H]DAP labeling may be made during the initial attachment steps prior to invasion. However, timed invasion experiments performed previously (data not shown) indicate that χ4335 was present intracellularly as early as 15 min postinoculation.

It is expected that some of these intracellular proteins belong to known stress regulons which are simply reacting to the change of environment (35). We are in the process of categorizing these by comparison to proteins synthesized by χ4335 in response to a variety of stresses such as heat shock and nutrient deprivation, etc. We are also looking at the initial response of χ4335 when it encounters the host cell prior to invasion. This is done by removal of bacteria in the supernatant at early time points after inoculation and analysis by the method described in this paper, which allows us to distinguish the synthesis of proteins required for recognition and/or attachment from those synthesized after invasion.

At present, DAP is only available commercially as a tritiated compound, which results in the need for gels to undergo extended exposure times on X-ray film. To further enhance incorporation we use the monolayers at approximately 18 h after incubation when intracellular levels of [^{3}H]DAP are highest. The results presented here show the cellular proteins of both Int-407 and χ4335 loaded together and later distinguished by autofluorography. To decrease the amount of host protein while increasing the amount of labeled (bacterial) protein that can be added to one gel, we have begun separating the bacteria from the host cells prior to analysis. While this step will result in the loss of any secreted proteins, we have chosen to address that issue in a separate study. This future study involves analysis of the supernatant from the infected monolayers to detect

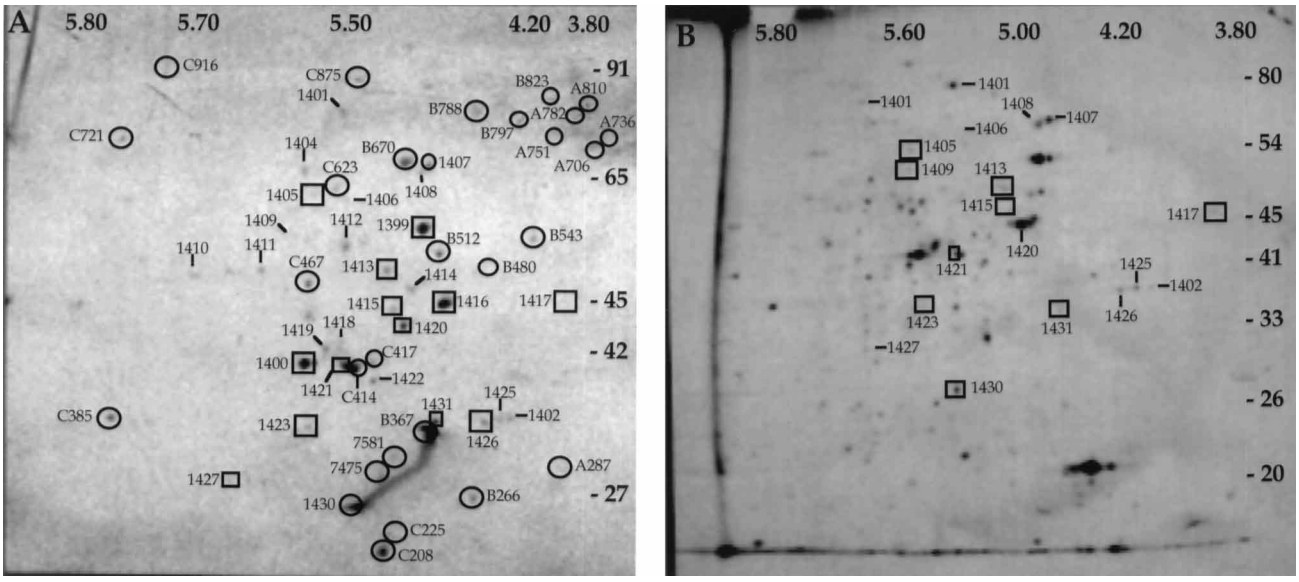

FIG. 8. 2-D SDS-PAGE and autofluorography of proteins synthesized by χ4335 during growth within Int-407 cells and labeled with either [^{3}H]DAP in the absence of cycloheximide (A) or with Tran^{35}S-label in the presence of cycloheximide (B). Squares represent proteins synthesized under both conditions, and circles represent proteins synthesized only in the absence of cycloheximide.

secreted bacterial protein. Secreted proteins would naturally be undetected by any method which involves separation of bacterial and host cells, and due to the increasing awareness of the importance of secreted proteins in the pathogenesis of various facultative intracellular pathogens such as *Salmonella* (14, 17), *Yersinia* spp. (25, 26), and *Shigella* spp. (2–4), we intend to extend our investigation to this group as well. The DAP-labeling protocol described here is very well suited to the detection of secreted proteins.

In summary, this paper presents a method whereby the proteins synthesized by *Salmonella* while growing intracellularly may be specifically labeled without cessation of host protein synthesis. A significant difference in the response of χ4335 to active versus inactive cells was evidenced by the group of 25 proteins synthesized only when the Int-407 cells were capable of interaction. We feel that this is a more relevant model as an inert host cell seems to represent a different challenge to *Salmonella* than one which is able to react. This method can be extended to the study of other gram-negative facultative intracellular pathogens such as *Shigella* and *Yersinia*, as well as *Mycobacterium* spp.

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