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Biochemical Phenotypes of *Salmonella Livingstone* Isolated from Humans, Animals and Feedstuffs in Sweden

By *M. Katouli, R. Wollin, A. Gunnarsson, I. Kuhn* and *R. Mollby*

Department of Bacteriology, Karolinska Institutet and Department of Bacteriology, the National Bacteriology Laboratory, Stockholm, and the National Veterinary Institute, Uppsala, Sweden.

Katouli, M.., R. Wollin, A. Gunnarsson, I. Kühn and R. Möllby: Biochemical phenotypes of *Salmonella Livingstone* isolated from humans, animals and feedstuffs in Sweden. Acta vet. scand. 1994, 35, 27-36. – *Salmonella Livingstone* is occasionaly isolated from humans, animals and feedstuffs in Sweden To follow the spread of infection and trace the source of isolates, adequate typing methods are needed We have developed an automated typing system based on biochemical fingerprinting of bacteria (the PhP system) for typing of different Salmonella serotypes. The system measures the kinetics of various biochemical reactions of bacteria grown in liquid medium in microtiter plates and uses numerical techniques to identify biochemical phenotypes (BPTs) among the tested strains. In the present study we used a set of 16 highly discriminatory tests to differentiate strains of *Salmonella* of serotype *Livingstone* and evaluated the system for its discriminatory ability using a collection of 34 unrelated human isolates of *S Livingstone.* We also used the system to investigate BPTs of 45 Livingstone strains isolated from animals and feedstuffs in Sweden between 1987 and 1991. Altogether 19 different BPTs were found among human isolate giving a diversity index (Di) of 0.930. In contrast, most strains isolated from animals and feedstuffs in Sweden belonged to 2 dominating BPTs (D_I = 0.704). One of these contained 17 strains mainly isolated during 1992 whereas the other contained 18 strains isolated between 1987 and 1991. None of the Swedish human isolates were identical to those of animals and feedstuffs These findings suggest that 2 different BPTs of *Salmonella Livingstone* strains are particularly common among animals and feedstuffs in Sweden and that they are not related to human cases of enteritis in this country. We also conclude that biochemical fingerprinting with the PhP system is a reliable and highly discriminatory method for detecting epidemic strains of *Salmonella Livingstone.*

biochemical fingerprinting.

Introduction

Salmonella enterica of serotype *Livingstone* was first isolated from cases of human enteritis in 1953 and characterized by Picton et al (1953). The organism is 1 of the 10 most frequently isolated serotypes in humans, animals and feedstuffs *(Eld et al.* 1991, *Badi et al.* 1989, *Mårtensson et al.* 1984). The organism has also been found in poultry and poultry feed in Sweden *(Eld et al.* 1991). Due to the economic losses for feed producing companies and outbreaks in poultry farms, it is important to provide evidence for the biologic and genetic relatedness of *S. Livingstone* strains as an aid in epidemiological investigations. The most widely used methods for characterization of

Salmonella strains include biotyping *(Duguid et al.* 1975, *Old et al.* 1985), phage typing *(Anderson et al.* 1977, *Guinee et al.* 1973), antimicrobial-susceptibility testing *(Anderson et al.* 1968, *Threlfall et al.* 1978) and colicin typing *(Barker,* 1980). The newer techniques of modern molecular biology have provided additional means of typing of *Salmonella* strains. These later methods include multilocus-enzyme electrophoresis of allo-enzymes *(Kapperud et al.* 1989), plasmid profile analysis *(Brunner et al.* 1983, *Schmidt et al.* 1982, *Riely et al.* 1983) and restriction-endonuclease analysis of plasmid or chromosomal DNA *(Eisenstein,* 1990, *Threlfall,* 1990). Advantages and disadvantages of these methods have also been evaluated in comparative studies *(Holmberg et al.* 1984, *Borrego et al.* 1992, *Rodrigue et al.* 1992). Among the more established phenotypic methods, biochemical profiles have been widely used to demonstrate phenotypic similarities or dissimilarities between isolates of *Salmonella* of different serotypes *(Barker et al.* 1988, *Reilly et al.* 1985, *Platt et al.* 1987). *Odongo et al.* (1990) developed a biotyping scheme for *S. Livingstone* consisted of 3 differentiating tests and used it to study a collection of strains isolated from animals and feedstuffs. Plasmid profile analysis and antimicrobial-susceptibility testing were then used to further differentiate the 4 primary identified biotypes *(Odongo et al.* 1990). This scheme, although it is of proven value, may not be sensitive enough since it utilizes only 3 differentiating tests.

The phene Plate (PhP) system is a computerized biochemical fingerprinting method which measures the kinetics of biochemical reactions of bacteria grown in liquid medium in microtiter plates. For each strain tested, it yields a set of quantitative data (the biochemical fingerprint) which is used to determine similarities among the tested strains with a personal computer program. The results are shown in the form of a dendrogram *(Möllby et al.* 1993, *Kühn et al.* 1990). The system has been evaluated for its reproducibility, discrimination and stability of the biochemical markers *(Katouli et al.* 1990, 1992a). It has also been used in epidemiological investigation of different *Salmonella* serotypes *(Katouli et al.* 1992b, 1992c, 1993). In the present study we optimized this system for typing of *S. Livingstone* and used it to investigate biochemical phenotypes of *Livingstone* strains isolated from animals and feedstuffs in Sweden between 1987 to 1992.

Materials and Methods

Bacterial strains

Two independent sets of *S. Livingstone* strains were examined. The first set comprised 34 epidemiologically unrelated isolates from sporadic cases of human enteritis. These strains had been isolated between 1990 to 1992 in 10 countries world wide and were selected from the collection held at the National Bacteriological Laboratory (NBL), Stockholm, Sweden (Table I). Strains had been stored on deep agar.

The second set, comprising 45 strains isolated from animals and feedstuffs in Sweden between 1987 to 1992, was obtained from the collection held at the National Veterinary Institute, Uppsala, Sweden. These strains had been stored in serum broth at –20°C since isolation (Table I).

Biochemical fingerprinting

The Phene Plate (PhP) system for biochemical fingerprinting of *Salmonella* serotypes (PhP-S plates) uses microplates with 3 parallel sets of 32 substrates. For each serotype a battery of tests with optimal discriminatory value has been selected out of these 32 tests and the computer program has been instructed to au-

Table I. Source, year of isolation and the number of *S Livingstone* strains from humans, animals and feedstuffs.

Unrelated strains from humans		Swedish strains from animals and feedstuffs		
Country	Year of isolation (number)	Place	Source	Year of isolation (number)
Denmark	1990 (1)	Borlange	Broiler	1989 (1)
Ethiopia	1991 (3)		Dog	1990 (1)
Indonesia	1990 (1)	Halmstad	Layer	1991 (7)
Mexico	1991 (1), 1992 (2)	Jonloping	Feedstuff	1987 (1)
Poland	1991 (2)	Karlstad	Dog	1990 (1)
Romania	1990 (1)	Kristianstad	Broiler	1991 (3)
Spain	1991 (11)		Feedstuff	1987 (2), 91 (1)
Sweden	1991 (7), 1992 (3)		Layer	1991 (1)
Tunisia	1990 (1)	Linkoping	Layer	1989 (1), 1991 (1)
Turkey	1990 (1)	Skara	Broiler	1989 (1), 1991 (2)
			Feedstuff	1987 (1), 1992 (1)
			Layer	1987 (4), 1992 (3)
		Sunne	Layer	1992 (4)
		Orebro	Layer	1992 (4)
		?	Broiler	1992 (1)
			Egg	1992 (2)
			Feedstuff	1987 (1), 1991 (1)

? denotes unknown source.

tomatically exclude the other tests from the calculations. Tests chosen for typing of Livingstone strains include: mann-d-lacton, D-xylose, melbiose, gentobiose, melezitose, dulcitol, tagatose, β-methyl-glucoside, 5-keto-gluconate, galactolacton, salicine, citrate, malinate, pyrovate, L-tartarate and ornithine. One colony of the strains to be tested was suspended in 10 ml Proteose Peptone (Difco) 0.1% w/v containing bromothymol blue 0.01% w/v and aliquots of 150 µl were inoculated to 32 wells in pre-prepared microtiter plates. To allow proper rehydration of substrates, plates were stored at 4°C overnight and incubated at 37°C the following morning. The absorbance $_{(A620)}$ of each reaction was measured at 4, 7, 24, and 48 h with a microplate reader. The absorbance values were transferred automatically to a personal computer and multiplied by 10, yielding scores ranging from 0 to 30 for each reaction; low values indicated acid (yellow) reactions and high values alkaline (deep blue) reactions. After the final reading, the mean value of 4 readings was calculated providing 32 different numbers ranging between 0 to 30 for each strain. The biochemical fingerprint for each strain was obtained after deletion of unwanted tests. Similarities between strains were calculated as correlation coefficients *(r)*, as described before *(Möllby et al.* 1993, *Kühn et al.* 1990) and clustered according to the unweighted-pair group method with arithmetic averages (UPGMA) *(Sneath & Sokal* 1973) yielding a dendrogram. Handling of data, including optical readings, was performed with the PhP software (BioSys inova, Stockholm, Sweden).

Reproducibility and identity level

Inter-assay reproducibility was calculated as mean correlation coefficient (r_{mean}) obtained from independent duplicate assays of 12 *S. Livingstone* isolates and the identity level (ID) was set at that reproducibility –2 standard deviations (SD) (95% confidence level). Strains with *r* values higher than the ID level used for the PhP-S system were assigned to the same biochemical phenotypes (BPT). BPTs with >1 isolate were called common (C) BPTs and those with only 1 isolate were called single (S) BPTs.

Discriminatory power

Two different methods of measuring discriminatory power of the system was used. The resolution index was calculated as $1\text{-}r_{mean}$ when all isolates within the studied population were compared *(Möllby et al.* 1993). *Diversity* was measured by Simpson's index of diversity (Di) *(Hunter* 1990) according to the formula:

$$Di = 1 - \Sigma [N_i(N_i-1)] / [N(N-1)]$$

where N_i is the number of isolates of the i^{th} phenotype, and N is the total number of isolates tested.

Results

Biochemical fingerprints of unrelated strains

The ID level of the PhP-S system for Livingstone strains was set at 0.980 after measuring the mean correlation of duplicate assays of 12 strains (i.e. 0.993) minus 2 SD (SD = 0.005). Using the above ID-level, 34 unrelated Livingstone strains were assigned to 5 common (C) BPTs, containing between 2-7 isolates, and 14 single (S) BPTs (Fig. 1). This gave a diversity index (D_i) of 0.930 and a resolution of 0.223.

Biochemical fingerprinting of Swedish strains

Among the 45 isolates from animals and feedstuffs, 2 C-BPTs and 10 S-BPTs were identified (Di = 0.771) (Fig. 2). The BPT C1 contained isolates from layers (n = 15), feedstuffs (n = 1) and eggs (n = 1) all except 4 isolated during 1992. In contrast, all strains in BPT C2 had been isolated between 1987 to 1991. This BPT contained isolates from feedstuffs (n = 5), broilers (n = 4) and layers (n = 9). At the similarity level of 0.85 all isolates were grouped into 3 main clusters (Fig. 2). Cluster A contained strains capable of fermenting galactonic-lactone ($galc^+$). Strains of BPT C1 (n = 17) within this cluster differed from the other BPTs mainly in their ability to ferment 5-ketogluconate (5-$keto^-$). Cluster B consisted of $galc^-$ strains with 1 strain (i.e. S9) also unable to ferment β-methyl glucoside (b $me.g^-$). The single isolate of cluster C differed from the others in many biochemical reactions (Fig. 2).

The unrelated set of strains, used for optimizing the system, contained 10 isolates from human cases of enteritis in Sweden during 1991 (n = 7) and 1992 (n = 3). To find out whether these isolates have the same BPTs as those from animals and feedstuffs, we compared these 2 groups. The results showed that all the Swedish human isolates belonged to BPTs totally different from those of animals and feedstuffs (Fig. 3).

Discussion

To be effective in epidemiological studies a typing method should have established precision and reproducibility, be stable over time and be sensitive enough to distinguish organisms that are similar but not identical. It is also desirable that the method is available and easy to perform specially when big outbreaks are to be investigated. Biochemical finger-

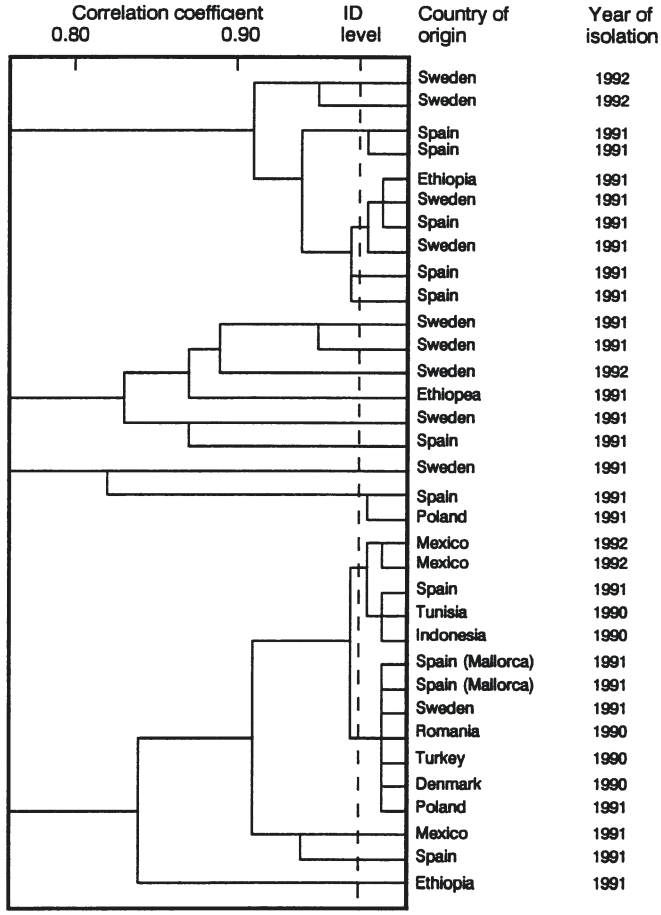

Figure 1. Dendrogram showing diversity and distribution of 34 unrelated human strains of *Salmonella Livingstone* by the PhP-S system.

printing with the PhP-S system proved to be an ideal method for typing of *Salmonella Livingstone.* In choosing tests for this scheme, we used the resolution index *(Möllby et al.* 1993). This index which is a special variable of numerical typing methods, shows the total discriminatory potential of all substrates used. If tests with identical values for all isolates are included in a scheme, the resolution index will decrease. For example, when we initially used 48 fermentation tests for typing 34 unrelated strains, a resolution index of 0.084 was obtained; strains were divided into 4 common and 10 single BPTs giving a diversity index of 0.829 (data not shown). However, after excluding tests with identical values for all *S. Livingstone* isolates the resolution index was increased to 0.223 and the diversity index to 0.930. Therefore, this index is a valuable tool for establishing an optimal set of tests for a

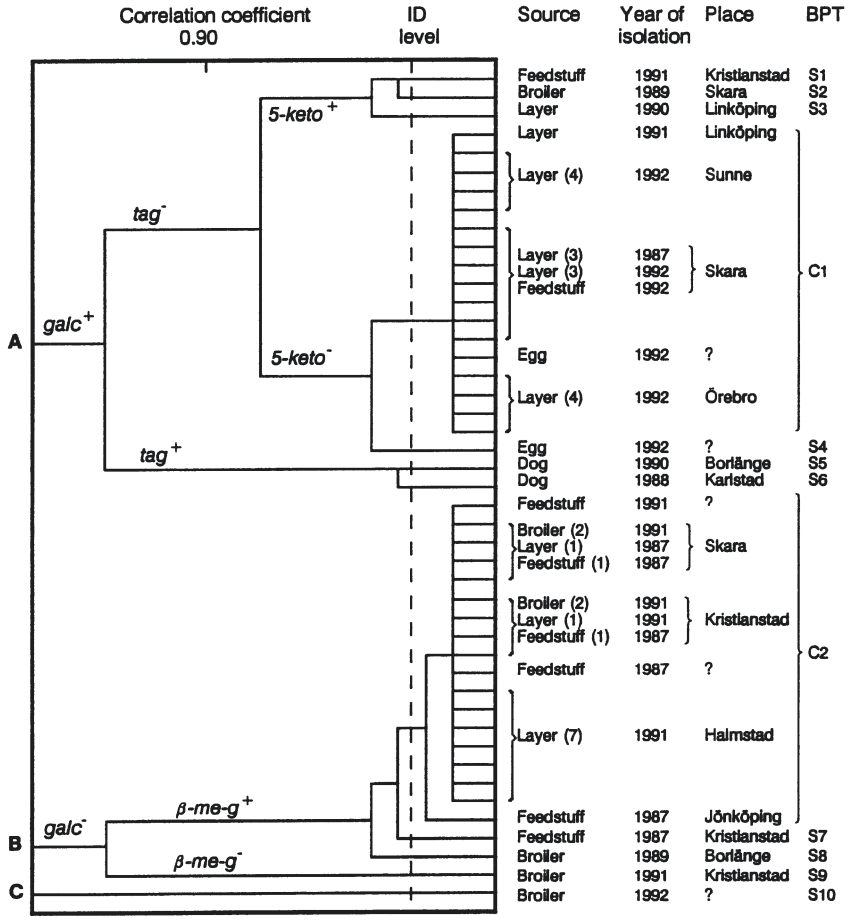

Figure 2 Biochemical phenotypes (BPTs) of 45 strains of *S.* Livingstone isolated from animals and feedstuffs in Sweden between 1987 to 1992. ID-level: Identity level; galc. galactonic-lacton, *tag:* tagatose; 5-keto: 5-keto-gluconate. ? = unknown

studied group of bacteria. Reproducibility is another important variable for assessing a typing method. It should ideally be 1, i.e the method should give the same results upon repeated testing. In this study reproducibility was calculated from inter-assay analysis of 12 duplicate samples and the identity level was set at the mean similarity between these duplicate assays –2 SD, giving 95% confidence level which is desirable for any typing system *(Hunter & Gaston* 1989).

The discriminatory power of a typing method, often expressed as the diversity index *(Hunter* 1990), measures the probability that 2 randomly selected strains will be assigned to different phenotypes by the actual typing method. A low diversity index means that many of the strains are of the same type or the typing system can not discriminate between them. Using a collection of epidemiologically unre-

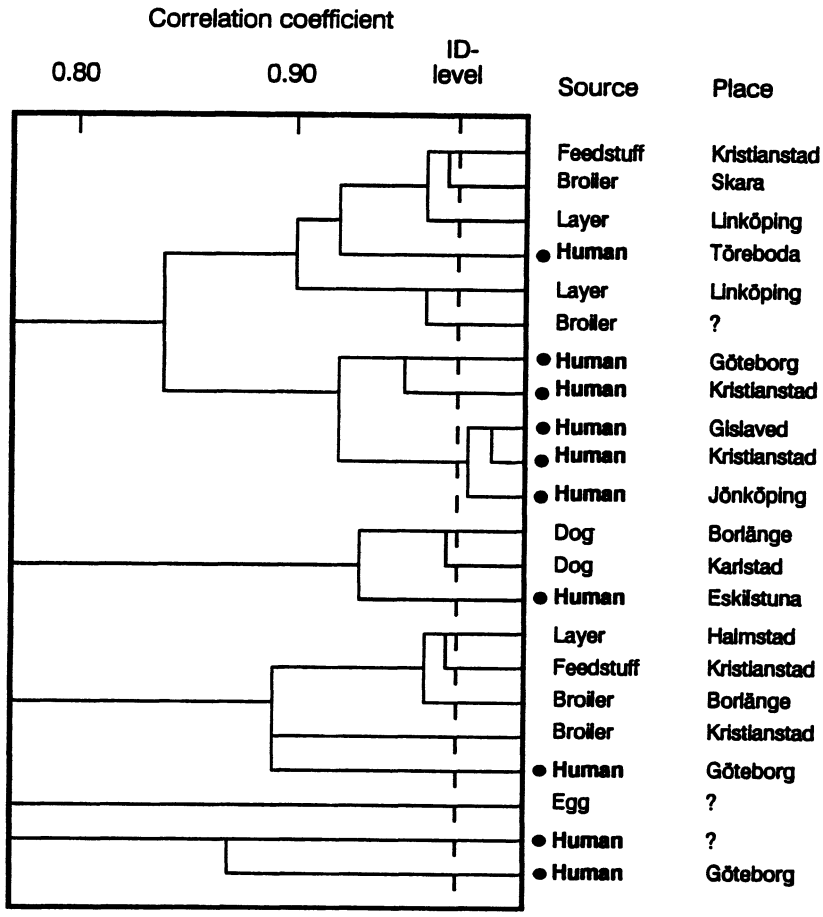

Figure 3. Comparison between biochemical phenotypes (BPTs) of *S.* Livingstone isolated from human cases of enteritises in Sweden (■) and those isolated from animals and feedstuffs in Sweden (One strain representing each BPT was selected from each group ; see Fig. 2). ? = unknown.

lated strains we obtained a diversity index of 0.930. In contrast, Swedish isolates from animals and feedstuffs had a diversity index of 0.704 which is a strong indication of an epidemiological relationship. Biochemical fingerprinting showed the presence of 2 major BPTs among this collection of strains. Other epidemiological data including year, place and source of isolation also supported our finding. Strains of these two BPTs differed from one another mainly in their ability to ferment galactonic-lactone (i.e. cluster A and B). This indicates that the ability to ferment galactonic-lactone (at least within the population tested in this study) may be an important factor in tracing the origin of *S. Livingstone* in animals and feedstuffs in Sweden. We previously used the PhP system together with phage typing and antimicrobial-susceptibility testing for detection of epidemic strains of *S. Typhimu-*

rium in Iran *(Katouli et al.* 1992*c*) and also found that those strains were divided based on their ability to ferment galactonic-lactone.

Another interesting finding in this study was that the Swedish isolates from humans had BPTs quite unlike to those isolated from animals and feedstuffs. Lack of a complete epidemiological data for the human isolates and their small number in this study make it rather difficult to draw a firm conclusion, but the fact that human isolates were not identical to those isolated from animals and feedstuffs, suggests that *S. Livingstone* strains found in animals and feedstuffs in Sweden may not be responsible for human enteritis in this country.

In conclusion, our data demonstrate that 2 different BPTs of *S. Livingstone* are responsible for a high proportion of infections among animals and contamination of feedstuffs in Sweden and that identical BPTs were not found among human isolates of *S. Livingstone* in this country. We also conclude that biochemical fingerprinting with the PhP-S system is an easy and reliable method for detecting major epidemic strains of *S. Livingstone.* The system is highly discriminatory and reproducible and can be used alone or together with other methods in epidemiological investigations of *S. Livingstone.*

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Sammanfattning

Biokemiska fenotyper av Salmonella Livingstone isolerade från Manniska, djur och fodermedel i Sverige.

Salmonella Livingstone isoleras ibland från människor, djur och djurfoder i Sverige. För att kunna kartlagga spridningen och identifiera ursprunget till

humana isolat ar det viktigt att ha adekvata typningsmetoder for denna organism. Vi har utvecklat ett automatiserat typningssystem som bygger på biokemisk fingerprintıng av bakterieisolat (PhP-systemet), och applicerat detta på bl.a. olika Salmonellaserotyper. Systemet utnyttjar mätningar av kinetiken för ett antal biokemiska reaktioner, som utfors på bakterier som odlas i mikrotiterplattor, och numeriska metoder anvands for att identifiera PhP-typer i det testade materialet. Vi har nu anpassat PhP-systemet for typning av *Salmonella Livingstone* och selekterat 16 diskriminerande reagens for denna Salmonella-typ. Systemet anvandes sedan for att typa en kollektion av 34 epidemiologiskt orelaterade humana Livingstone isolat, samt for att studera 45 Livingstone-stammar, isolerade i Sverige mellan 1987 och 1991 från djur och djurfoder. Bland de humana isolaten hittade vi sammanlagt 19 olika PhP-typer, och diversitetsindex var 0.930. Bland isolaten från djur och djurfoder dominerade 2 typer, och diversitetsindex var bara 0 704. Den ena av dessa dominerande PhP-typer bestod av 17 isolat, främst från 1992, och den andra av 18 isolat från 1987-1991. Ingen av de svenska humana isolaten var identisk med de som återfanns bland djur och djurfoder. Våra data tyder alltså på att de båda Livingstone-stammarna som var vanliga bland djur och djurfoder i Sverige ej ar identiska med de som isolerats från manniskor. Vi har också visat att biokemisk fingerprintıng med PhP-systemet ar en diskriminerande metod att kartlägga spridningen av Livingstone-stammar.

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Reprints may be requested from: M. Katouli, Department of Bacteriology, Karolinska Institutet, P.O. Box 60400, S-10401 Stockholm, Sweden.