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Characterization of a collection of *Enterobacter sakazakii* isolates from environmental and food sources

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Abstract

Enterobacter sakazakii has emerged as a rare cause of neonatal meningitis, septicemia and enterocolitis. Contaminated infant milk formula (IMF) has been identified as one infection route. A small number of clinical outbreaks have been epidemiologically linked to IMF contaminated post-pasteurization during manufacture and/or mishandled when reconstituted. Currently no agreed standardized typing protocol has been developed to trace *E. sakazakii*. The objectives of this study were to apply biochemical and genetic methods to characterize 51 environmental and food *E. sakazakii* isolates and 6 *E. sakazakii* type strains. Isolates were presumptively identified using biochemical profiles based on API 20E and ID32E methods and by culture on differential selective Druggan Forsythe Iversen (DFI) agar. Identification was subsequently confirmed by real time polymerase chain reaction (PCR).

All but one of the isolates was identified as *E. sakazakii* by biochemical profiling. One isolate was identified as *Escherichia vulneris* by ID 32E and as *Pantoea agglomerans* by API 20E. All isolates produced green/blue colonies on DFI medium characteristic of this organism. Real time PCR could differentiate between *E. sakazakii, Enterobacter* spp. and other *Enterobacteriacae*. Analysis of RAPD banding patterns revealed 3 major clusters of *E. sakazakii*. There was a large degree of diversity noted amongst the remaining isolates. Our findings indicate that RAPD may be applied as a useful and reliable tool for direct comparison of *E. sakazakii* isolates providing traceability through the infant formula food chain.
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Keywords: *Enterobacter sakazakii*; RAPD typing; Neonatal meningitis

1. Introduction

Enterobacter sakazakii (*E. sakazakii*) is an opportunistic pathogen causing meningitis, septicaemia and enterocolitis in neonates (Bar-Oz et al., 2001; Muytjens and Kollee, 1982). Preterm, low-birth-weight or immunocompromised infants exposed to *E. sakazakii* are at particular risk (Lai, 2001). Mortality rates of 10–80% have been described and survivors often suffer from neurological sequelae (Gallagher and Ball, 1991; Lai, 2001; Ries et al., 1994). Clinical outbreaks of infection in neonatal intensive care units associated with contaminated infant milk formula (IMF) have been reported (Block et al., 2002; van Acker et al., 2001).

E. sakazakii is an ubiquitous organism (Kandhai et al., 2004). The source of *E. sakazakii* and vehicle of transmission is not always clear however infant formula has been epidemiologically implicated as the source of *E. sakazakii* in several clinical cases (Clark et al., 1990; Simmons et al., 1989; van Acker et al., 2001; Weir, 2002). The source of contamination of IMF is thought to include a broad range of dry blended raw material, together with possible environmental sources associated with the production environment. To minimize possible contamination of IMF both the raw materials and the production environment must be constantly monitored. Molecular subtyping has been applied as a useful tool to facilitate surveillance, tracing routes from a source to an infected individual. Possible sources include isolates from raw materials and/or the manufacturing environment. Importantly, this approach makes it possible to distinguish a persistent environmental strain that

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could intrinsically contaminate IFM from extrinsic isolates introduced post-manufacturing.

Generally methods based on phenotype analysis are acknowledged to be unreliable due to the unstable expression of the corresponding marker(s). For this reason DNA-based protocols offer an attractive alternative. Furthermore DNA fingerprinting allows for a direct comparison of isolates in outbreaks. Previously, non-standardized DNA fingerprinting protocols have been applied to *E. sakazakii* (Clark et al., 1990; Nazarowec-White and Farber, 1999). Reported methods used include ribotyping, pulsed-field gel electrophoresis (PFGE) and random amplification of polymorphic DNA (RAPD) (Clark et al., 1990; Nazarowec-White and Farber, 1999). These molecular tools facilitate the traceback of outbreak isolates from clinical sources to the contaminated batch of powdered infant milk formula and/or the manufacturing environment. In addition, they are useful tools to target control strategies and reduce the risk of transmission. The only comparison between various molecular subtyping protocols in the literature was reported by Nazarowec-White and Farber (1999). These authors characterized 18 *E. sakazakii* isolates using three molecular typing methods including ribotyping, PFGE, and RAPD.

In this paper we report the characterization of a collection of food and environmental *E. sakazakii* isolates. Our investigations included presumptive identification based on biochemical profiles and culture on selective media. Identification of each isolate was confirmed by a modified real time PCR protocol. Finally, RAPD was used to generate DNA fingerprinting profiles creating a database of *E. sakazakii* isolates for comparison purposes.

2. Materials and methods

2.1. Bacterial isolates and culture media

Fifty-seven *E. sakazakii* isolates were investigated in this study (Table 1). 51 of these were food and environmental isolates. Six *E. sakazakii* NCTC strains were included as controls in all experiments. All isolates were initially cultured on Tryptic Soy Agar [TSA] (Oxoid Hampshire, UK) and then onto DFI agar (Oxoid) to confirm α-glucosidase activity (Iversen et al., 2004a). All isolates were preserved on cryostat beads (Mast Diagnostics, Merseyside, United Kingdom) at −80 °C.

2.2. Biochemical identification

Isolates were identified biochemically using API 20 E kits and ID 32 E panels according to the manufacturer's instructions (BioMerieux, Mary l' Etolie, France).

2.3. DNA isolation

Total DNA was prepared using the Wizard Genomic DNA purification kit (Promega, Madison, WI). DNA concentrations were determined spectrophotometrically as previously described (O'Mahony et al., 2005). The integrity of the purified template DNA was assessed by conventional agarose gel [1%, (w/v)] electrophoresis and DNA preparations were stored at 4 °C.

2.4. Real-time PCR

A real time PCR targeting the *dnaG* gene on the macromolecular synthesis (MMS) operon of *E. sakazakii* and was used to confirm the identification of the *E. sakazakii* isolates (Seo and Brackett, 2005).

2.5. RAPD analysis

Molecular typing was performed by random amplified polymorphic DNA (RAPD) as previously described (Nazarowec-White and Farber, 1999). All isolates were examined in duplicate for reproducibility and in a single assay to reduce inter-test variability. Amplified products were visualized following electrophoresis through a conventional 1.5% (w/v) agarose gel stained with ethidium bromide (10 mg/ml) in a 0.5X Tris-Borate-EDTA (TBE) buffer. Gels were visualized and photographed using the Gel Doc 2000 system (BioRad, Hercules, CA). DNA fingerprints were stored as tagged image file format (TIFF) files and imported into the BioNumerics software (Version 4.0; Applied-Maths, Sint-Martens-Latem, Belgium) where dendrograms were created using the DICE coefficient and the unweighted pair group method with arithmetic mean (UPGMA). The discriminatory power of RAPD was assessed by the modified index of diversity suggested by Hunter (1990).

In addition, a minimum spanning tree was constructed using the BioNumerics software package. A binary data set was created from the RAPD fingerprinting files by calculating a global band matching table (position tolerance of 1% and an optimization of 1%). This band matching table was used to calculate a minimum spanning tree where the creation of hypothetical nodes was allowed.

2.6. Statistical analysis

The degree of correlation between RAPD cluster analysis and phenotypic biochemical characteristics were examined by categorizing the ability of the RAPD clustered isolates to ferment inositol and palatinose sugars as indicated by API tests. Two-by-two contingency tables of data categorized by both RAPD cluster and individual sugar fermentation characteristics were devised. Differences amongst calculated proportions were examined using Fischer's exact test, and the Kappa statistic was calculated to quantify interrator agreement. Sugar fermentation results were also combined in series and in parallel and correlations with RAPD clusters examined using similar categorical analytical methods. Statistical analyses were performed using Graphpad Version 4.0 (Graphpad Software, San Diego, California).

3. Results

3.1. Bacterial isolates

All colonies produced yellow pigmentation on TSA and appeared as blue/green colonies confirming constitutive expression of α-glucosidase on DFI agar (data not shown).

Table 1
E. sakazakii isolates used in this study

Isolate Identifier	Isolate Source	API 20E Profile	ID 32E Profile	Inositol Api20 E	Inositol ID 32 E	Palatinose ID 32 E	RAPD Profile
ES 105	II	3305173	34276763050	−	−	−	A
ES 205N	IV	3305373	34276767210	+	+	+	A
ES 206N	IV	3305373	34276767210	+	+	+	A
ES 207N	IV	3305373	34276767210	+	+	+	A
ES 208N	IV	3305373	34276767050	+	+	−	A
ES 228N	IV	3305373	34276767250	+	+	+	A
ES 255N	IV	3305373	34276767250	+	+	+	A
ES 304N	IV	3305373	34276767250	+	+	+	A
ES 306N	IV	3305373	34276767250	+	+	+	A
76	I	3305173	34276363250	−	−	+	B
88	I	3305173	34276363250	−	−	+	B
89	I	3305173	34576761250	−	−	+	B
90	I	3305173	34276763250	−	−	+	B
91	I	3305173	37276763050	−	−	−	B
95	I	3305173	34076763040	−	−	−	B
96	I	3305173	34076763040	−	−	−	B
98	I	3305173	34076763040	−	−	−	B
75	III	3305173	34276363010	−	−	−	C
ES 97	I	3305173	34274763050	−	−	−	C
ES 99	I	3305173	34076763050	−	−	−	C
172	I	3305173	34276763250	−	−	+	C
ES 254N	IV	3305173	34276767250	−	+	+	C
ES 305N	IV	3305173	34276763250	−	−	+	C
ES 36	Type strain	3305173	34276767210	+	+	+	−
ES 37	Type strain	3305173	34274767210	−	+	+	−
ES 43	Type strain	3305173	34276763210	−	−	+	−
ES 44	Type strain	3305373	34276767210	+	+	+	−
ES 45	V	3305173	34276763200	−	−	+	−
ES 49	III	3305173	34276763251	−	−	+	−
ES 50	III	3305373	34276763251	+	−	+	−
ES 52	Unknown	3305373	nd	+	nd	nd	−
ES 53	Unknown	3305373	34076763210	+	−	+	−
ES59	Unknown	3305373	34276763251	+	−	+	−
ES 73	II	3305373	34276767250	+	+	+	−
ES 74	V	3305173	34276363040	−	−	−	−
ES 79	II	3305373	34276767050	+	+	−	−
ES 80	II	3305373	34276767050	+	+	−	−
ES 82	II	3305373	34076767250	+	+	+	−
ES 83	VII	3305173	34276763250	−	−	+	−
ES 92	Unknown	3305173	34276763050	−	−	−	−
ES 93	I	3305173	75276763250	−	−	+	−
ES 94	I	3305173	36276763250	−	−	+	−
ES 101	II	3305173	34076761050	−	−	−	−
ES 102	II	3305173	34276763040	−	−	−	−
ES 103	II	3305373	34274767050	+	+	−	−
ES 104	II	3305373	34276767050	+	+	−	−
ES 106	II	3305173	34276763040	−	−	−	−
ES 110	II	3305373	35276767050	+	+	−	
ES 112	II	3305173	30276763050	−	−	−	−
ES 135	I	1004153	04474543051	−	−	−	−
ES 162	I	3305173	34276763050	−	−	−	−
ES 187	III	3347373	03477367251	+	+	+	−
ES 191	II	3305173	34276763050	+	+	+	−
ES 195	VII	3305373	34276767250	+	+	+	−
ES 201	VI	3305373	74276763250	+	−	+	−
11467	Type strain	3305373	nd	+	nd	nd	−
08155	Type strain	3305373	nd	+	nd	nd	−

The 57 isolates were grouped into two categories based on their geographical origins. Forty were cultured from sources in Europe: 36 were isolated from various sources designated (I: $n=15$, II: $n=13$, III: $n=4$, V: $n=2$, VII: $n=2$). The remaining 4 European isolates were of unknown environmental origin. Eleven isolates belonging to groups IV ($n=10$) and VII ($n=1$) were obtained from sources from food and environmental sources in Australasia. In addition six type strains were included in the RAPD analysis (Fig. 1a) and biochemical profiling. nd=not done.

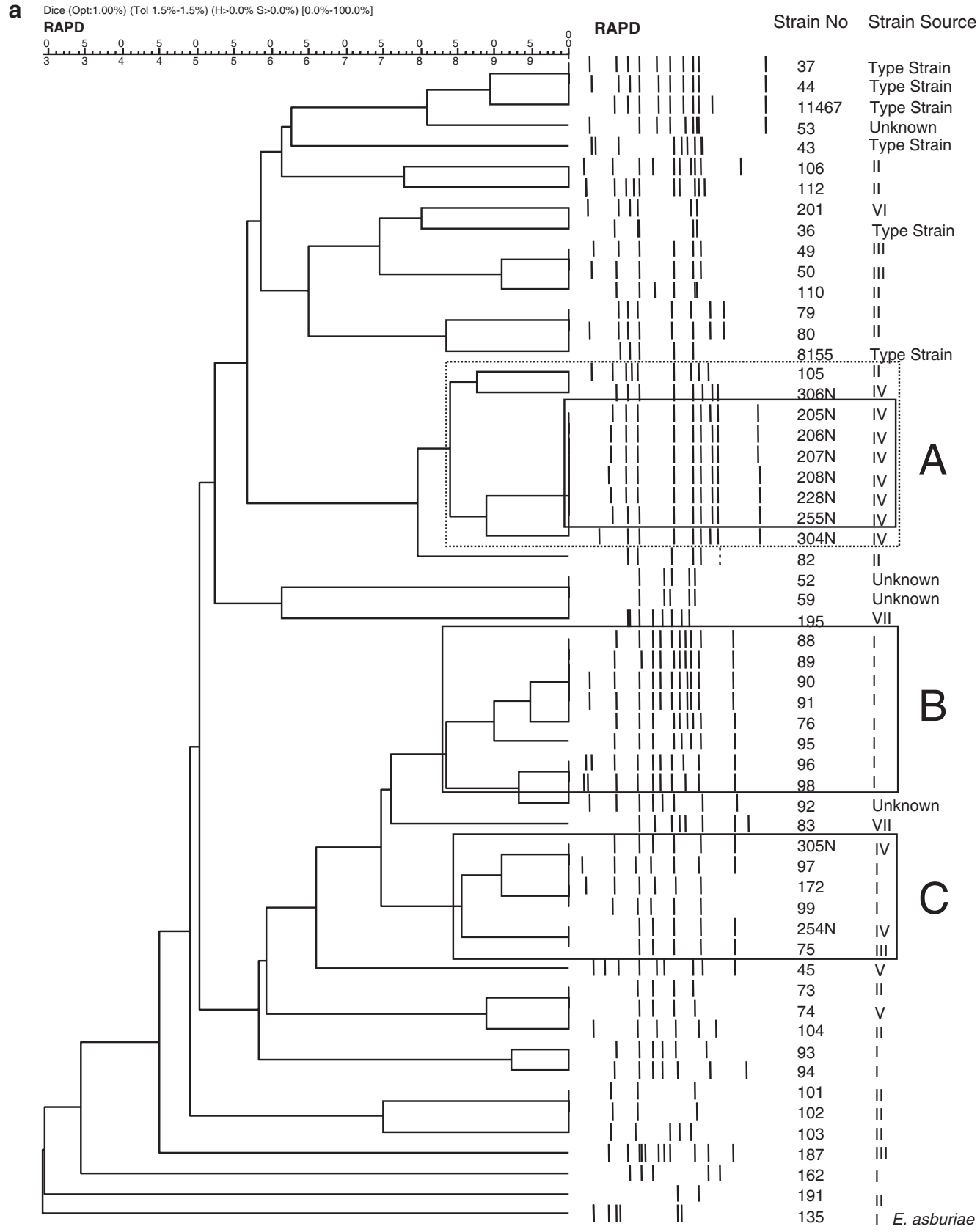

Fig. 1. (a) A dendrogram obtained from cluster analysis (Dice similarity coefficient; unweighted pair-group average method) of RAPD patterns of the 56 isolates. Clusters A, B and C are indicated. Strain identifier and the corresponding source where known are indicated. (b) Chromosomal DNA digested with Xba1 and separated by pulsed field gel electrophoresis. Lanes 1–8 group IV isolates from RAPD cluster A (205N, 206N, 207N, 208N, 228N, 255N, 304N, 306N) lanes 9 and 10 isolates from cluster B (305N and 254N, respectively).

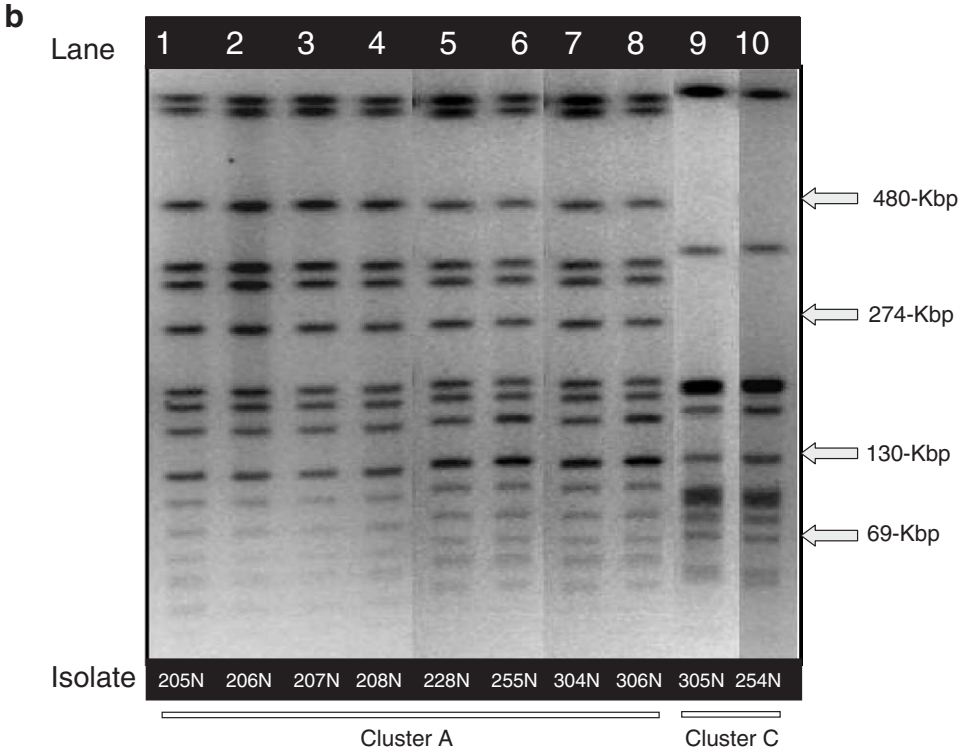

Fig. 1 (*continued*).

3.2. Biochemical identification profiles

The API 20E biochemical profiles obtained identified all but one isolate as *E. sakazakii* with percentage identification values ranging from 91.1% through 99.2%. All of the isolates identified as *E. sakazakii* had very similar biochemical profiles differing in the fermentation of a single sugar (inositol). A single isolate (ES-135) was misidentified as *Pantoea agglomerans* with a biochemical identification value of 71.1%. Similarly the ID32E protocol also identified 56 strains as *E. sakazakii*. Again ES-135 was misidentified; in this case as *Escherichia vulneris*. In this assay format, the panel of biomarkers was more discriminatory compared to API 20E, as 30 profiles differing in 18 sugars were observed. Isolate ES-135 was subsequently identified by 16S rRNA sequencing as *Enterobacter asburiae* (data not shown).

The biochemical profiles determined by both methods in this study were distributed over both geographical groups. Analysis of the range of biochemical reactions specifically identified 5 indicators that provided increased discrimination, and these included palatinose, sodium malonate, lipase, sodium pyruvate and inositol (Table 1). Fermentation of the sugar substrates inositol and palatinose showed the greatest variation and both were examined in greater detail to determine whether fermentation of either sugar correlated with RAPD cluster analysis.

In addition to the discriminatory tests above a number of false negative reactions were observed with both biochemical tests (data not shown). These data did not affect our observations or conclusions.

3.3. Real-time PCR

To further confirm the identity of this collection, a modification of the original real-time PCR assay design described by Seo and Brackett (2005) was applied to purified template genomic DNA from all study isolates. In addition to the study collection (of 57 isolates), 25 non-*E. sakazakii* were included (comprising *E. cloacae, P. aglomerans, E. aerogenes* and *E. gergoviae*). Sixty non-*Enterobacter* spp. including *Citrobacter freundii, C. divergens, Klebsiella pneumoniae, K. oxytoca, Salmonella typhimurium, S. infantis, E. coli, Campylobacter coli. C. jejuni, Staphylococcus aureus,* and *Clostridium difficile* were also evaluated by this method. Fifty-six of the 57 study isolates tested positive, confirming their identity as *E. sakazakii*. All other *Enterobacter* spp. and non-*Enterobacter* spp. similarly tested negative using this protocol (data not shown).

3.4. RAPD profiling and genomic cluster analysis

Template DNA was purified from all 57 study isolates along with a control group of isolates for comparison purposes. All were analyzed by RAPD and a quantitative assessment of the genomic relationships was made based on the BioNumerics software output. Each isolate produced banding patterns consisting of 3 to 11 amplified products, ranging in size from approximately 350 to 2600 bp. Three clusters designated A through C were identified based on a quantitative analysis of the banding patterns obtained (Fig. 2a). Cluster A contained six isolates from the Australasia region that were highly related

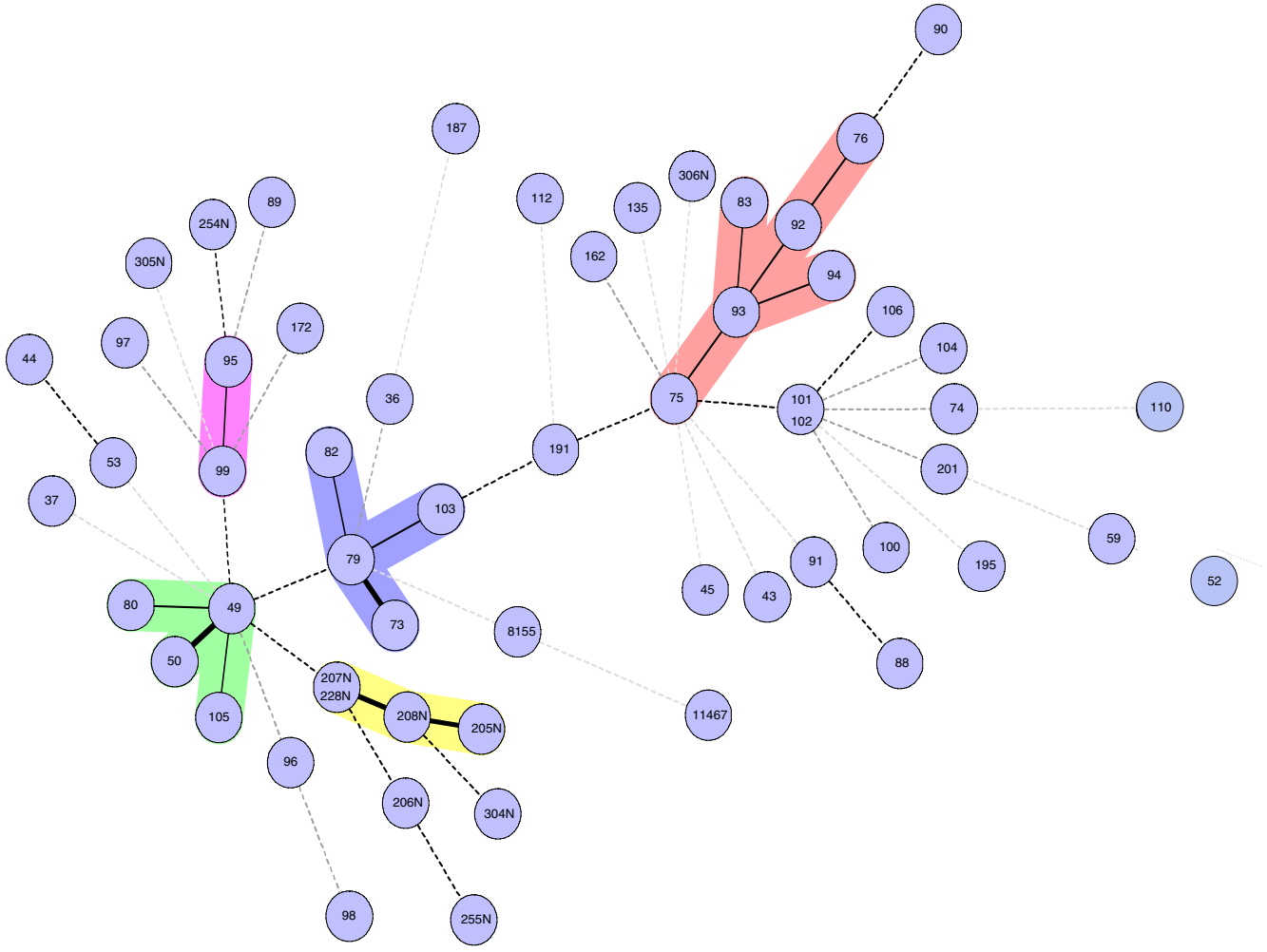

Fig. 2. A minimum spanning tree of the RAPD fingerprinting data. The number within each circle indicates the isolate number (see Table 1). An empty or white circle indicates a hypothetical node. The length of the branches is proportional to the distance between the types and the thickness, dotting and graying of the branch lines also indicate the distance between the nodes. Each group is indicated by a different color.

clustering at 100% (indicated by the solid line box in Fig. 2a). Three additional isolates were highly related to these 6 clustering at 88% (and included ES-304N) and 84% (isolates ES-305N and ES-105), respectively (denoted in Fig. 2a by the broken line box). In cluster B there were 8 isolates that were 84% similar. All isolates in this group were cultured from a common European environmental source denoted as I (in Table 1). Cluster C contained six isolates that were 84% identical, despite the fact that two isolates were obtained from Australasia along with the four isolates from Europe. Isolates ES-254N and ES-75 showed identical RAPD patterns but originate from Europe and Australasia, respectively. Moreover, there was no known epidemiological connection between the sources of these two isolates.

The remaining 34 isolates had diverse RAPD banding profiles with similarities of less than 80% and these were considered to be genetically unrelated. Interestingly, isolate ES-135 (*E. asburiae*) was amongst the most diverse of the isolates demonstrating only 35% similarity to clusters A–C (Fig. 1a). A discriminatory power of 97.6% was calculated for this technique. All isolates in the RAPD clusters A through C were investigated by PFGE. Genomic DNA was prepared as previously described (Daly et al., 2004) and digested with *Xba1*. Fig. 1b shows the profiles from the ten group IV isolates from Australasia. Eight of these isolates belong to RAPD cluster A and were found to be indistinguishable using PFGE (Fig. 1b, lanes 1–8) while 2 belonging to RAPD cluster C and these were also indistinguishable by PFGE (lanes 9 and 10, Fig. 1b). Furthermore, isolates ES-254N and ES-75 produced identical PFGE DNA profiles and similarly had indistinguishable RAPD profiles, despite originating from different continents (data not shown). Using BioNumerics software, a minimum spanning tree was created based on RAPD banding patterns to further explore the phylogenetic relationships between isolates (Fig. 2). Although the majority of the isolates appeared unrelated, the minimum spanning tree identified several different groupings among this set of isolates. In particular the yellow group (indicated in Fig. 2) including the surrounding branches corresponded to isolates from Cluster A of the RAPD dendrogram (Fig. 1a). *E. sakazakii* isolate ES-254N and-305N were outside this Australasia cluster, further confirming their genetic unrelatedness.

3.5. Correlation between RAPD analysis and sugar fermentation

According to the bioMerieux API 20E definitions 75% of *E. sakazakii* isolates ferment inositol. Similarly, the ID 32 manual describes the number of inositol fermenting *E. sakazakii* strains as 50% with palatinose positive isolates determined to be 82%. In our culture collection 24 of the 57 isolates (43%) were inositol positive by the API 20E method (INO 20 column in Table 1). These included 9 isolates from the Australasia region 8 of which belonged to RAPD cluster A and 15 isolates from Europe that did not belong to any of the major RAPD clusters (A–C). Using the ID 32E system (INO 32 column in Table 1) inositol was positive for 20 of the 57 isolates (36%); 10 isolates from the Australasia region (8 from RAPD cluster A and 1 from cluster C) and 10 from Europe. Palatinose was positive for 30 of the 57 isolates (54%); 11 isolates from the Australasia region and 19 from Europe). These included 14 isolates belonging to major RAPD clusters (7, 4 and 3 isolates in clusters A, B and C respectively) and 16 isolates that did not belong to clusters A–C.

Isolates that were positive for both INO 32 and INO 20 were significantly more likely to belong to RAPD Cluster A, than in another cluster ($p=0.0011$).

Similarly, isolates in RAPD cluster B were significantly less likely to ferment INO ($p=0.0042$). The respective Kappa statistics indicated only a poor to moderate agreement beyond chance (0.441 and 0.308), respectively. There was no correlation between inositol fermentation and RAPD cluster C or palatinose fermentation and any of the RAPD clusters.

4. Discussion

E. sakazakii has emerged as a rare cause of life-threatening neonatal meningitis, septicemia and enterocolitis (Farmer et al., 1980). The organism has been detected in dry infant milk formula products; and the improper handling of these formulae has been implicated in several clinical cases. The U.S. Food and Drug Administration (FDA) recommended procedure for the isolation of this organism from IMF is based on the original work of Muytjens and Kollee (1982), and uses standard isolation methods for Enterobacteriaceae, with additional selection of yellow-pigmented organisms, and subsequent biochemical identification. However, this method is not selective for *E. sakazakii* and could lead to false negative and positive results (Lehner et al., 2004).

To facilitate the detection of *E. sakazakii* several media have been formulated. These take advantage of the key biochemical characteristic of this bacterium, the production of α-glucosidase. Recently a chromogenic agar was developed by Druggan-Forsythe-Iversen (DFI agar) where a chromogenic substrate 5-bromo-4-chloro-3-indolyl-α-D-glucopyranoside (X-αGlc) is used as an indicator of α-glucosidase activity (Iversen et al., 2004a). Oh and Kang (2004) described a differential selective media containing a fluorogenic substrate 4-methyl-umbelliferyl-α-D-glucopyranoside which similarly detects α-glucosidase activity. The same fluorogen is also included in a differential non-selective medium [LBDC] described by Leuschner and Bew (2004). However, α-glucosidase activity is not restricted to *E. sakazakii* strains (Lehner et al., 2004). Moreover, recent studies based on 16S rRNA and *hsp60* indicated that culture and biotyping lack the necessary discriminatory power required to confidently identify distinct phylogenetic lineages amongst *E. sakazakii* isolates (Iversen et al., 2004b; Lehner et al., 2004). Detection and identification by culture methods is labour intensive and requires both enrichment together with long incubation times. The recommended FDA approach requires 5 days to confirm a positive result. Future application of suitable molecular microbiology methods will offer an additional approach to culture and biochemical analysis useful for the rapid detection and identification of bacterial pathogens. Furthermore, molecular sub-typing protocols facilitate surveillance providing traceability of organisms from clinical and environmental settings.

In this study we used biochemical and molecular typing techniques to investigate 51 presumptive *E. sakazakii* strains isolated from food and environmental sources and 6 type strains. Biochemical profiling using two different commercially available methods identified all but one isolate as *E. sakazakii*. Real time PCR confirmed the identification of all *E. sakazakii* and this modified method was capable of distinguishing *E. sakazakii* from both non-*sakazakii Enterobacter* spp. and other *Enterobacteriaceae*. We found that RAPD could distinguish between isolates grouping them into clusters while demonstrating the diverse nature of the remaining isolates studied.

Culture of the 57 isolates on TSA and DFI media resulted in 1 false positive result, strain EA-135 was subsequently identified by 16S rRNA sequencing. Using the biochemical profiles all *E. sakazakii* isolates (56) were identified correctly, however a number of false negative and positive reactions were observed with both test methods. Furthermore, both biochemical identification systems require 24 h to complete. To determine the discriminatory potential of the biochemical tests we evaluated the fermentation of inositol and palatinose sugars. While significant differences amongst the calculated proportions was observed between inositol fermentation and RAPD clusters A and B, the small numbers examined in this study along with the poor level of agreement beyond chance makes meaningful interpretation difficult. Further analysis of larger numbers of isolates would be required to more accurately assess these observed trends. Based on our observations in this study, comparison of biochemical with RAPD profiles indicated that biochemical tests were poorly discriminating thereby limiting their combined utility for tracing isolates in an industrial and/or nosocomial setting. Discrepancies between biochemical identification tests further highlighted the problems encountered when such tests are based on the detection of phenotypic markers alone and whose expression are often unstable. In contrast, the real-time PCR approach correctly identified all 56 *E. sakazakii* isolates within 90 min, following the isolation of a pure culture.

There are few studies that have addressed molecular typing and the detection of *E. sakazakii*. A study by Clarke et al. (1990) used ribotyping to analyse 30 *E. sakazakii* from the environment, raw milk, and finished IMF identified eight distinct ribotypes. A second study by Nazarowec-White and Farber (1999)

characterized 18 Canadian *E. sakazakii* isolates from food and clinical sources using ribotyping, RAPD, and PFGE. That study is the only published comparison of typing methods and concluded that PFGE and RAPD gave greater discriminatory power compared to ribotyping.

Our findings indicate that RAPD is capable of discriminating among a collection of *E. sakazakii*. Profiling data can be generated quickly at a low cost in terms of consumables, technical expertise and equipment. However, establishing a standardized international library of RAPD profiles could prove difficult due to the lack of reproducibility associated with inter-laboratory comparison of RAPD protocols. A more standardized approach could be developed based on standardized PFGE similar to those protocols developed for other enteric organisms under the PulseNet system. This development would provide a standardized database facilitating global comparisons of different *E. sakazakii* isolates. Nevertheless, RAPD is a useful molecular tool providing rapid analysis of isolates in a local context. Moreover, other studies underway in our laboratory suggest that RAPD will be at least as discriminatory as PFGE in typing these isolates.

In conclusion, molecular methods can support conventional microbiology to trace *E. sakazakii* in the infant food chain. These approaches will contribute to the protection of public health.

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