

Towards a Typing Strategy for *Arcobacter* Species Isolated from Humans and Animals and Assessment of the *In Vitro* Genomic Stability

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Abstract

Arcobacter species have a widespread distribution with a broad range of animal hosts and environmental reservoirs, and are increasingly associated with human illness. To elucidate the routes of infection, several characterization methods such as pulsed-field gel electrophoresis (PFGE), amplified fragment-length polymorphism, and enterobacterial repetitive intergenic consensus (ERIC)-PCR have already been applied, but without proper validation or comparison. At present, no criterion standard typing method or strategy has been proposed. Therefore, after the validation of PFGE, those commonly applied typing methods were compared for the characterization of six human- and animal-associated *Arcobacter* species. With a limited number of isolates to be characterized, PFGE with restriction by *KpnI* is proposed as the first method of choice. However, ERIC-PCR represents a more convenient genomic fingerprinting technique when a large number of isolates is involved. Therefore, a first clustering of similar patterns obtained after ERIC-PCR, with a subsequent typing of some representatives per ERIC cluster by PFGE, is recommended. As multiple genotypes are commonly isolated from the same host and food, genomic plasticity has been suggested. The *in vitro* genomic stability of *Arcobacter butzleri* and *A. cryaerophilus* was assessed under two temperatures and two oxygen concentrations. Variability in the genomic profile of *A. cryaerophilus* was observed after different passages for different strains at 37°C under microaerobic conditions. The bias due to these genomic changes must be taken into account in the evaluation of the relationship of strains.

Introduction

SINCE THE DESCRIPTION OF THE GENUS, *Arcobacter*-associated human and animal illness has been reported worldwide (Vandamme *et al.*, 1992b). Predominantly the species *A. butzleri*, and also to a lesser extent the species *A. cryaerophilus* and *A. skirrowii*, are implicated in enteritis and occasionally septicemia in humans (Vandenberg *et al.*, 2004; Wybo *et al.*, 2004). Clinical symptoms are similar to a *Campylobacter jejuni* infection but a more persistent, watery diarrhea has been reported (Kiehlbauch *et al.*, 1991; On *et al.*, 1995; Hsueh *et al.*, 1997; Yan *et al.*, 2000; Woo *et al.*, 2001; Lau *et al.*, 2002; Fernandez *et al.*, 2004; Vandenberg *et al.*, 2004; Wybo *et al.*, 2004). Infection probably occurs through the consumption of contaminated food, in particular poultry products, pork, beef, and drinking water (Jacob *et al.*, 1998; Ho *et al.*, 2006; Van Driessche and Houf, 2007). Other potential risk factors are contact with pets and person-to-person transmission (Vandamme *et al.*, 1992a; Houf *et al.*, 2008; Fera *et al.*, 2009). *Arcobacter* differs from closely related

Campylobacter spp. by the abilities to grow at lower temperatures and in higher oxygen concentrations (Vandamme *et al.*, 1992b).

One of the main challenges in *Arcobacter* research is to identify the different transmission routes. Various studies reported multiple species and moreover several strains present in the same host and on food products (Houf *et al.*, 2002; Van Driessche *et al.*, 2004; Van Driessche *et al.*, 2005), hampering correct identification of vectors and reservoirs. Currently, genotypic methods are commonly applied, but not all have been validated (e.g., pulsed-field gel electrophoresis [PFGE]) or used (e.g., multilocus sequence typing) for all *Arcobacter* species (Hume *et al.*, 2001; Son *et al.*, 2006; Miller *et al.*, 2009; Giacometti *et al.*, 2013). At present, no criterion standard typing method or strategy has been proposed. Furthermore, strain or genotype delineation is currently arbitrary without guidelines specified. Besides the interpretation challenges due to different typing methods, the possibility of genomic rearrangements, as also described in *C. jejuni* (Wassenaar *et al.*, 1998; Ridley *et al.*, 2008;

Leblanc-Maridor *et al.*, 2011), may have their basis in the large strain diversity. However, whether or not these rearrangements are induced by *in vitro* manipulation is currently unknown.

The aims of this study were to validate PFGE for its use with six human- and animal-associated *Arcobacter* species, and to propose a typing strategy for arcobacters based on the comparison of PFGE with two other previously validated and commonly used typing methods: amplified fragment gel polymorphism (AFLP) and enterobacterial repetitive intergenic consensus (ERIC)-PCR. Finally, the *in vitro* genomic stability of *A. butzleri* and *A. cryaerophilus* was evaluated by multiple cultivations under different temperature and atmosphere conditions.

Materials and Methods

Bacterial strains and growth conditions

For the validation and comparison of the typing methods, 46 *A. butzleri*, 33 *A. cryaerophilus*, 33 *A. skirrowii*, 38 *A. thereius*, 11 *A. cibarius*, and 12 *A. trophiarum* isolates as well as 25 *Arcobacter* collection strains (Table 1) were included. The *Arcobacter* isolates were obtained from food, human stool, and feces from food-producing animals by the Department of Veterinary Public Health, Ghent University, Belgium, using *Arcobacter* selective isolation methods for food and feces (Houf *et al.*, 2001; Van Driessche *et al.*, 2003; Houf and Stephan, 2007). For the *in vitro* genomic stability assays, 24 arcobacters were included: 6 *A. butzleri* and 7 *A. cryaerophilus* strains recently isolated from pig feces (De Smet *et al.*, 2011), 5 *A. butzleri* and 4 *A. cryaerophilus* isolates recently isolated from human stool (Saint-Lucas Hospital, Ghent), as well as 2 *A. cryaerophilus* collection strains LMG 9904^T and LMG 9947.

All strains and isolates were grown on Mueller–Hinton agar (CM 0337, Oxoid, Basingstoke, UK) supplemented with 5% defibrinated horse blood (E&O Laboratories Ltd., Bonnybridge, Scotland) and incubated for 48 h at 30°C under microaerobic conditions by evacuating 80% of the normal atmosphere and introducing a gas mixture of 8% CO₂, 8% H₂, and 84% N₂ into the jar.

TABLE 1. ARCOBACTER COLLECTION STRAINS INCLUDED

Strains	Reference numbers
<i>A. butzleri</i>	LMG 10828 ^T (= ATCC 49616), LMG 10900, LMG 11932, LMG 6620, LMG 10223, LMG 11120, LMG 9906, LMG 9910
<i>A. thereius</i>	LMG 24486 ^T
<i>A. cibarius</i>	LMG 21996, LMG 21997
<i>A. cryaerophilus</i>	LMG 9865, LMG 9904 ^T , LMG 10210, LMG 9863, LMG 9065, LMG 9947, LMG 9867, LMG 10230, LMG 10237
<i>A. skirrowii</i>	LMG 6621 ^T , LMG 9912, LMG 14985, LMG 10234
<i>A. trophiarum</i>	LMG 25534 ^T

LMG, Culture Collection of the Laboratory for Microbiology, Ghent University, Belgium; ^T, type strain; ATCC, American Type Culture Collection.

Validation of PFGE

Different protocols, based on the PulseNet protocol for *Campylobacter jejuni*, were tested (PulseNet 2009: <http://www.cdc.gov/pulsenet/protocols.htm>). The two restriction enzymes, *EagI* and *KpnI* (New England Biolabs Inc., Beverly, MA) previously used in *Arcobacter* characterization studies were included (Hume *et al.*, 2001; Son *et al.*, 2006). Other restriction enzymes were evaluated *in silico* using the software program FastPCR (Kalendar *et al.*, 2011) on the whole genome sequence of the *A. butzleri* strain ATCC 49616 (= LMG 10828^T), deposited in GeneBank[®] (Miller *et al.*, 2007). To evaluate the typeability, repeatability, and discriminatory power, 25 *Arcobacter* collection strains (Table 1) were cultivated in triplicate, and a cell suspension with an optical density of 1.5 measured at 610 nm with a biophotometer (Eppendorf AG, Hamburg, Germany) was prepared in 10 mL of phosphate buffered saline (PBS) (Sigma-Aldrich, Irvine, Ayrshire, UK). Plugs were prepared by adding the adjusted cell suspension (400 µL) in a sterile microcentrifuge tube containing 20 µL of a 20 mg/mL stock solution of proteinase K (Invitrogen, Carlsbad, CA), to which an equal volume of 1% SeaKem Gold agarose (Cambrex, Rockland, ME) was added. Plug slices were digested for 5 h at 37°C with 40 U of the different restriction enzymes in single digestion reactions. In order to assess the reproducibility, the 25 strains were re-cultivated twice at different points of time, and the corresponding fingerprints were compared.

Subsequently, 173 *Arcobacter* isolates, comprising the six human- and animal-related species and identified by an *Arcobacter* species-specific multiplex-PCR (Doudidah *et al.*, 2010) were characterized with the validated PFGE protocol, using the most discriminatory restriction enzymes.

Comparison of different typing techniques

Preparation of DNA templates. The 25 collection strains and 173 *Arcobacter* isolates used in the validation of PFGE were cultivated as described above. A cell suspension of each strain with an optical density of 0.074 ± 0.002 (measured at 660 nm [Biophotometer, Eppendorf]), corresponding to approximately 10⁷ colony-forming units/mL was prepared in 10 mL of PBS. Template DNA of each strain was extracted from a 0.5-mL cell suspension by the guanidinium thiocyanate method (Pitcher *et al.*, 1989). Five microliters of each DNA template was size separated by electrophoresis to evaluate their integrity. The concentration as well as the purity of each DNA template was determined spectrophotometrically. The DNA templates were adjusted to 50 ng/µL and stored at –20°C until further analysis.

AFLP. For AFLP analysis, the validated protocol for *Arcobacter* species by Debruyne *et al.* (2010) was applied, using *HindIII* and *HhaI* as restriction enzymes (New England Biolabs Inc.) and analysis performed by capillary gel electrophoresis using an automated 16-capillary sequencer (3130xl Genetic Analyzer, Applied Biosystems, Carlsbad, CA).

ERIC-PCR. ERIC-PCR assay was exactly performed according to the previously validated protocol for *Arcobacter* by Houf *et al.* (2002).

Assessment of the *in vitro* genomic stability of *A. butzleri* and *A. cryaerophilus*

In a first experiment, the *A. cryaerophilus* strains LMG 9947 and LMG 9904^T, and four isolates—*A. butzleri* R643, *A. cryaerophilus* R637 and R671 from pig feces, and *A. cryaerophilus* R206 from human stool—were included. Strains were cultivated on Mueller–Hinton agar supplemented with 5% defibrinated horse blood at 30°C under microaerobic conditions for 48 h. One colony was picked and recultivated on Mueller–Hinton agar plates under the same conditions. To avoid subjective selection of specific colonies, further subcultivations were performed by picking a loopful of bacterial growth, striking them on blood plates, and incubating them at 30°C and 37°C, each under aerobic and microaerobic conditions. The strains were further subcultivated in the same way twice a week for 8 weeks (= 16 passages). At the start and on even-numbered passages, PFGE was performed as described above. A second experiment was performed based on the results obtained in the first experiment but with an at-random selection of 10 *A. butzleri* and 8 *A. cryaerophilus* strains isolated from humans and pigs, as well as again strain R671. The strains were now cultivated for three generations at 30°C and 37°C under microaerobic conditions as described above and subsequently characterized by PFGE.

Data analysis. The PFGE and ERIC profiles were analyzed with GelCompar 3.0 (Applied Maths, Sint-Martens-Latem, Belgium). For PFGE, the *Salmonella* serovar Braenderup H9812 strain was restricted with *Xba*I enzyme and used as a molecular size standard. Computer-based normalization and interpolation of the ERIC-PCR profiles was performed using TrackIt™ 100-bp DNA Ladder (Cat. No. 10488-058, Invitrogen). AFLP profiles were collected with the Data Collection software v 3.0 (Applied Maths). Profiles were imported in BioNumerics v 4.61 (Applied Maths) for further analysis using the CrvConv filter. Similarities between fingerprints were calculated using the Dice coefficient for PFGE, and Pearson correlation coefficient for ERIC-PCR. AFLP normalization was performed using the internal LIZ-600 standard, after which similarities between profiles were determined by the Pearson and product moment correlation coefficient. PFGE, ERIC, and AFLP dendrograms were constructed by numerical analysis of obtained profiles using the unweighted-pair group method using arithmetic means algorithm. Profiles were automatically assigned to groups according to (restriction) pattern similarities, which were visually controlled and edited if needed. The relationship between *Arcobacter* strains was expressed as a percentage of similarity.

Unrelated strains were included to compare the discriminatory ability of the three typing methods using the Simpson's index of diversity (Hunter and Gaston, 1988; Simpson, 1949). This index indicates the probability of two strains sampled randomly from a population belonging to two different types. Furthermore, the Adjusted Rand (*AR*) and the adjusted Wallace (*AW*) were calculated in order to compare the congruence between typing methods (Severiano *et al.*, 2011). Online tools were used for calculation of these indexes (<http://darwin.phylviz.net/ComparingPartitions/index.php?link=Tool#>).

Results

Validation of PFGE

Besides the two restriction enzymes *Eag*I and *Kpn*I previously used in *Arcobacter* typing (Hume *et al.*, 2001; Son *et al.*, 2006), only the restriction enzymes *Nru*I and *Stu*I generated the required minimum of 10 and a maximum of 30 fragments after *in silico* restriction of the *A. butzleri* genome ATCC 49616 (van Belkum *et al.*, 2007). In the characterization of the 25 *Arcobacter* collection strains, fingerprints for all strains were obtained with all enzymes, but the enzymes *Eag*I and *Stu*I were shown to be less discriminative, since for some strains low numbers of fragments (<5) were obtained (data not shown). Furthermore, they generated identical fingerprints for some strains of different *Arcobacter* species (data not shown). For further experiments, the restriction enzyme *Kpn*I was selected as first choice as it generated an average number of 15.5 fragments (ranging from 10 to 30), in contrast to an average of 8 bands (ranging from 5 to 13) with the *Nru*I (data not shown). Optimal analyses were achieved with PFGE carried out in a 1% SeaKem (wt/vol) gold agarose (Cambrex) in a PFGE apparatus (Bio-Rad, Hercules, CA) for 18 h with a pulse time of 6.676–13.68 s for *Kpn*I (Son *et al.*, 2006), and 5.2 s–42.3 s for *Nru*I under a constant temperature of 14°C, a gradient of 6 V/cm, an angle of 120°, and a range of 30 kbp–400 kbp. A 100% repeatability and reproducibility of the PFGE protocol, both with *Kpn*I and *Nru*I, was obtained.

Comparison of different typing techniques

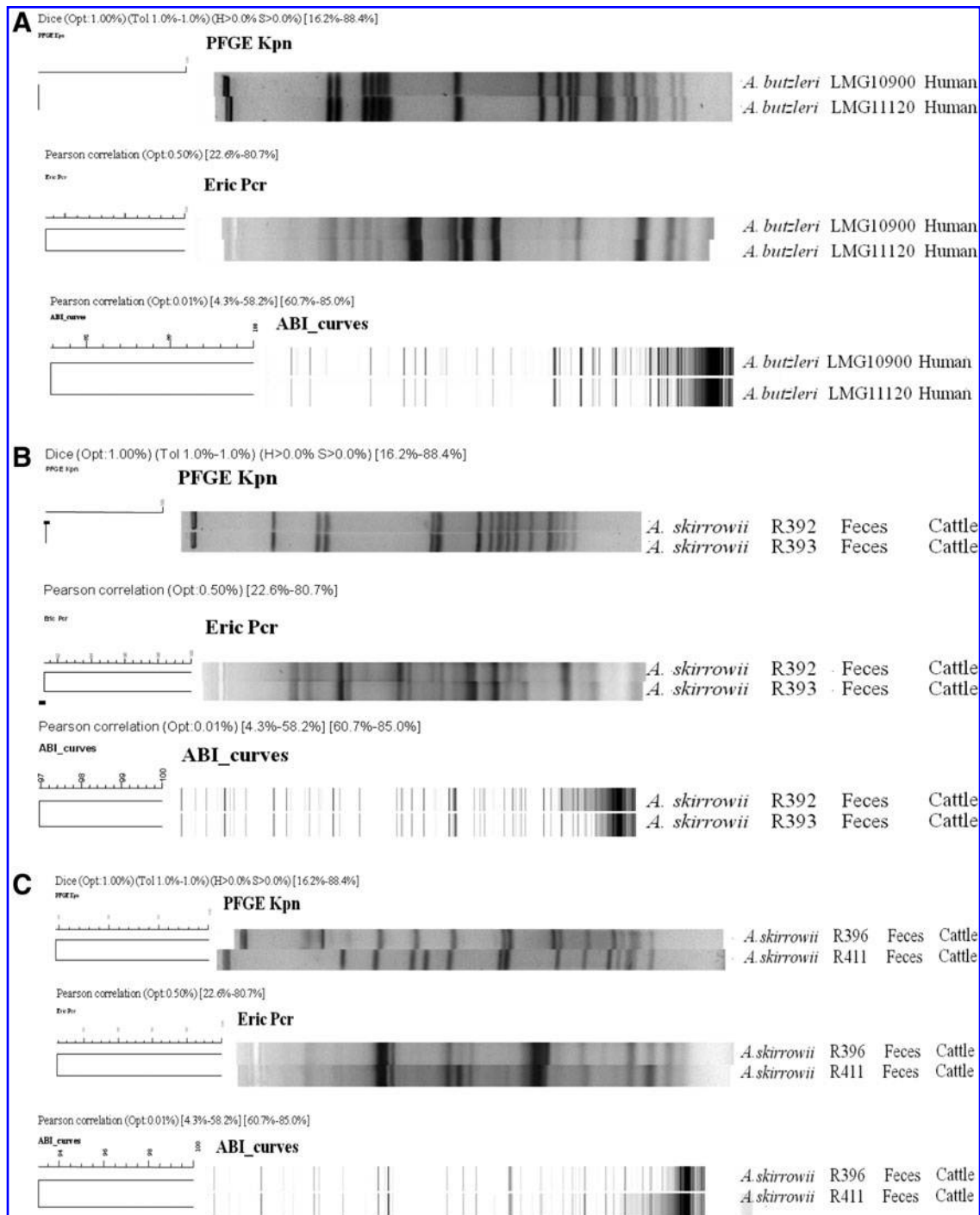
Similarities between fingerprints were calculated as described above, with an optimization of 1.0% and a position tolerance of 1.0% for PFGE. For ERIC-PCR, an optimization of 0.5% and 0.5% position tolerance was applied. Due to the high resolution of capillary migration, an optimization and a position tolerance of 0.01% was used for AFLP (Debruyne *et al.*, 2010).

All 198 *Arcobacter* collection strains and isolates could be characterized by ERIC-PCR and AFLP. With PFGE, no fragment patterns were obtained for one *A. cryaerophilus*, one *A. skirrowii*, and nine *A. thereius* isolates using restriction with both *Kpn*I and *Nru*I. The inability to type all *Arcobacter* strains resulted in a 94.5% typeability for PFGE, to 100% for ERIC-PCR and AFLP. The discriminatory powers of the three typing methods were determined by calculating the Simpson's index of the unrelated *A. butzleri* and *A. cryaerophilus* isolates typed by these methods. Due to the large diversity of *Arcobacter* isolates, all three methods showed high discriminatory power, with a discriminatory index of 1. *AR* and *AW* coefficients were also calculated to explore the correlation between the typing methods; however, the same result of 1 was obtained. Weighing the results of the three typing methods for the unrelated *Arcobacter* isolates showed comparatively high discriminatory power, revealing no difference between the methods.

In general, ERIC-PCR generated the lowest number of bands with an average between 12 and 15 bands per profile for the six *Arcobacter* species, except for *A. thereius* and *A. trophiarum*, which generated in AFLP the lowest number of bands (i.e., six and nine bands per AFLP profile, respectively). The number of bands generated by PFGE and AFLP

was quite similar, except for *A. thereius* and *A. trophiarum*, as explained above. Cluster analysis per method of the DNA patterns showed no clear distinction at *Arcobacter* species level. A large heterogeneity between the isolates was observed, with related or identical strains clustering more closely with all three methods than nonrelated strains. However, no clear relationship between sources of origin or between isolates from animal and human origin was observed. Isolates with similar profiles generated by one method were compared with the corresponding profiles obtained by the other two

typing methods. For *A. butzleri*, two isolates from the Italian outbreak (Vandamme *et al.*, 1992a) (LMG 10900 and 11120) with an identical PFGE profile also generated high similar (>97.57%) ERIC-PCR and AFLP profiles (Fig. 1A). Furthermore, two *A. skirrowii* isolated from the feces of related cows (R392, R393) showed similar PFGE profiles and highly similar AFLP (96.95% similarity), but less similar (>91%) ERIC-PCR profiles (Fig. 1B). The isolates R396 and R411, isolated from feces of cattle from the same farm, generated both ERIC-PCR and AFLP profiles that were more than 91%



(continued)

FIG. 1. Comparison of PFGE, ERIC-PCR, and AFLP patterns of different *Arcobacter* species.

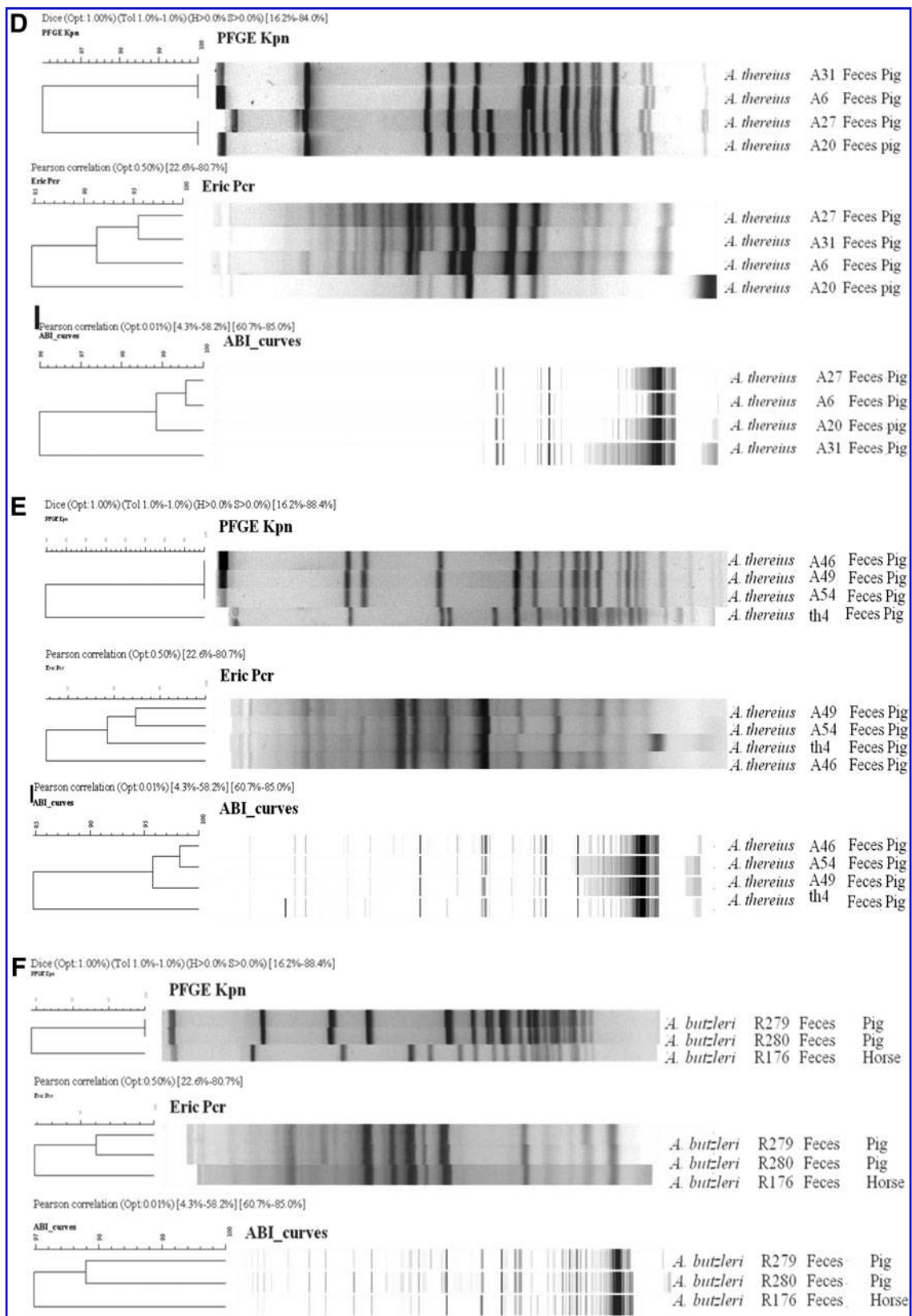


FIG. 1. (Continued)

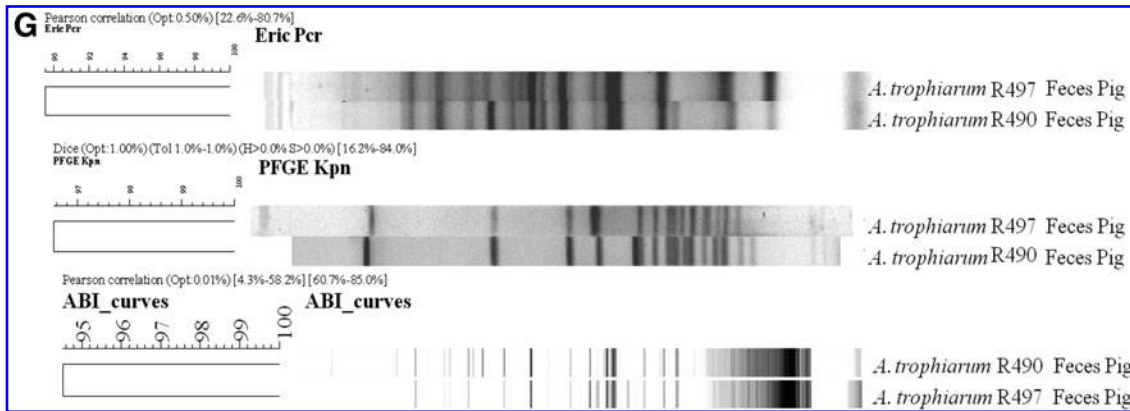


FIG. 1. (Continued)

and 93% similar, respectively. In contrast, PFGE patterns showed only 85% similarity (Fig. 1C).

Examining four closely related *A. theaeus* isolates (A6, A20, A27, and A31) from pig feces, PFGE showed profiles with more than 96% similarity (Fig. 1D) (De Smet *et al.*, 2011). The isolates A20 and A27 generated different PFGE profiles in comparison to the other profiles, due to the presence of an additional band. In AFLP, all four isolates were similar when applying a 96% cut-off value. In addition, isolates A27 and A6 generated more than 99.5% similar AFLP patterns. In contrast to the PFGE and AFLP patterns, ERIC-PCR analyses showed a similarity of 95% for isolates A27 and A31, but a lower similarity between those two and isolates A6 and A20 (Fig. 1D). Also for the related *A. theaeus* isolates A46, A49, and A54 from pig feces (De Smet *et al.*, 2011), identical PFGE profiles were obtained, and ERIC-PCR and AFLP discriminated them at a 98% and 95% similarity level, respectively. Although a similarity of 97.8% was observed with isolate Th4 by ERIC-PCR, both AFLP and PFGE profiles showed a discordance for the same isolate compared to the A46, A49, and A54 profile (<85%) (Fig. 1E).

A. butzleri R176 and R280, isolated from horse and pig feces, respectively, generated PFGE profiles with less than 70% similarity, while more than 97% similarity was obtained by AFLP and ERIC analyses (Fig. 1F). *A. trophiarum* strains with highly similar PFGE profiles (96.8%; strains R490, R497) showed almost 90% and 95% similarity by ERIC-PCR and AFLP, respectively (Fig. 1G).

Assessment of the in vitro genomic stability of *A. butzleri* and *A. cryaerophilus*

The genomic stability of six *Arcobacter* strains was evaluated by PFGE after 16 subcultivations under two different temperature and two oxygen conditions. In general, PFGE profiles of all strains were identical throughout the experiment under aerobic conditions both at 30°C and 37°C. However, variations in PFGE profiles were seen under microaerobic conditions at 37°C, except for *A. butzleri* R643 and *A. cryaerophilus* LMG 9904T (data not shown). From passage 4 on, an additional band in *A. cryaerophilus* strain R206 and a displacement of a single fragment in

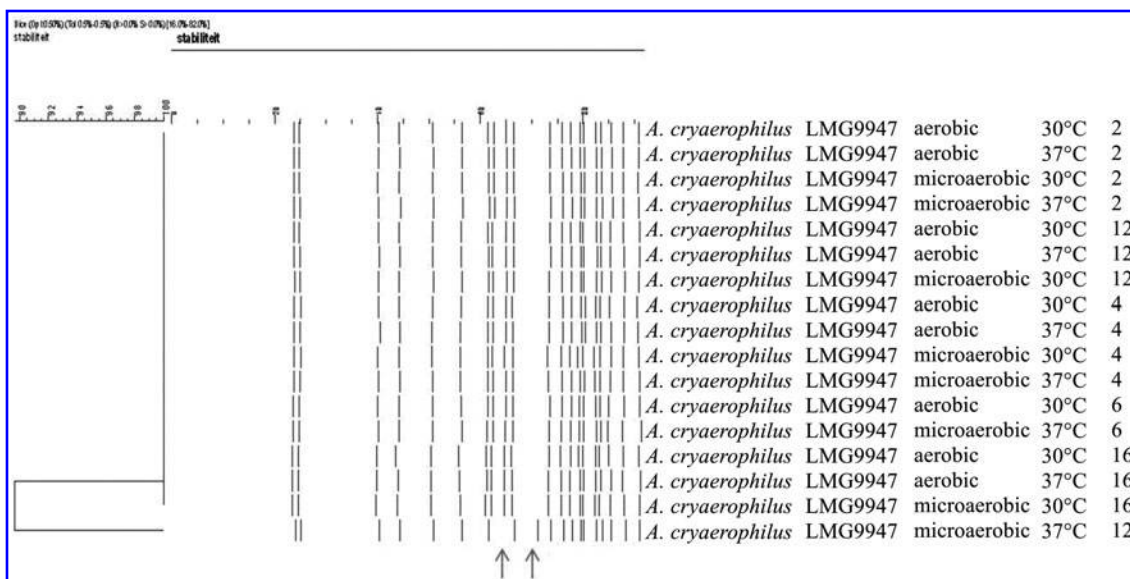


FIG. 2. UPGMA dendrograms from PFGE profiles using restriction with *KpnI* showing the assessment of the in vitro genomic stability of *A. cryaerophilus* strain (LMG 9947) isolated from bovine fetus.

A. cryaerophilus strain R637 were detected. For strain LMG 9947, two bands disappeared while a smaller band became visible from passage 12 on (Fig. 2). From passage 2 on, a large band disappeared and an additional smaller band became visible in the profiles of *A. cryaerophilus* strain R671 at 37°C, both under microaerobic and aerobic conditions. The strains showing changing PFGE patterns were also screened by ERIC-PCR, but no differences in ERIC-PCR profiles could be observed.

This first experiment showed that exposure to environmental conditions of 30°C and 37°C together with microaerobic oxygen levels can induce changes in PFGE patterns. To assess whether this genomic instability already occurs at earlier passages, 10 *A. butzleri* and 8 *A. cryaerophilus* strains were cultivated for 3 generations at 30°C or 37°C under microaerobic conditions. No variations in band profiles were obtained for any of the *A. butzleri* strains (data not shown). With *A. cryaerophilus*, identical fingerprints were obtained for all strains, except for strain R861, where in both passages identical PFGE profiles at 30°C were obtained, while two new PFGE profiles were generated at 37°C, with a difference of five and seven bands on passages 1 and 3, respectively (data not shown).

Discussion

In the present study, the molecular typing methods ERIC-PCR, PFGE, and AFLP were compared in their ability with six *Arcobacter* species for the first time. Analysis of a set of 198 *Arcobacter* isolates confirmed once again the large strain diversity existing in the six *Arcobacter* species. No correlation between typing patterns from strains isolated from humans, animals, or food was noticed. These findings are similar to those in the multilocus sequence typing study, where no association with either host or geographical source was observed (Miller *et al.*, 2009).

In general, with a limited number of isolates to be characterized and taking into account the recommendations by van Belkum *et al.* (2007), PFGE with restriction by *KpnI* is proposed as the first method of choice. When identical fragments for apparently unrelated isolates occur, a second PFGE analysis with the restriction enzyme *NruI* is recommended. The reason no fingerprints were obtained for some strains could be due to an excessive restriction by endogenous endonucleases or by DNA methylation activities (van Belkum *et al.*, 2007). However, as PFGE is time consuming and labor intensive, ERIC-PCR represents a more convenient genomic fingerprinting technique, and has already been used for a limited number of species in the past (Houf *et al.*, 2002; Aydin *et al.*, 2007) and in an *A. butzleri* outbreak investigation (Vandamme *et al.*, 1993). This method has the advantage of being rapid and easy to perform compared to the other two typing methods. The reproducibility of this method is often questioned, though, as shown in previous studies, the fingerprints as such cannot be exchanged but the interpretation is reliable. Nevertheless, delineation of genotypes or strains remains challenging when PCR-based methods are applied. No guidelines for genotype delineation have been proposed, and a fixed percentage above which isolates are considered identical is not possible. Within each fingerprint cluster generated by PCR-based methods, three classes of fingerprints are obtained: isolates clearly

different, isolates clearly identical, and a group of fingerprints with minor variations in the presence or intensity of some amplicons. The intensity of the amplicons is often not interlaboratory reproducible, in contrast to their presence or absence (Houf *et al.*, 2002; Van Driessche *et al.*, 2004; Van Driessche *et al.*, 2005). Therefore, in the case that genotype delineation is strictly necessary, isolates differing in one or two amplicons in PCR-generated fingerprints should be further examined by PFGE.

Though analysis by AFLP generates comparable results as obtained by ERIC-PCR, it is more expensive, demands specific equipment, and has been shown to be less discriminative for both *A. theaeus* and *A. trophiarum*, and therefore, less recommended for *Arcobacter* typing.

In the present study, genomic instability due to *in vitro* manipulation was only observed in some strains of *A. cryaerophilus*, especially under microaerobic conditions at 37°C, partly explaining the enormous heterogeneity reported in this species. During transmission and the colonization of the intestinal tract of humans and animals, *Arcobacter* undergoes different stress factors comparable to those in the experiments, such as temperature and oxygen concentration. The *Arcobacter* genome seems to be exposed to relatively rapid genomic changes *in vitro*, but data on *in vivo* conditions, as performed for *Campylobacter* (Wassenaar *et al.*, 1998; Ridley *et al.*, 2008; Leblanc-Maridor *et al.*, 2011), are lacking. One should keep in mind that these differences in profiles were not picked up by ERIC-PCR, meaning that the variation in PFGE profiles are probably caused by (point) mutations. Nevertheless, the present study showed once again that the large heterogeneity is not artificial or completely induced by laboratory actions of the typing methods applied. This still leaves the question on the origin of this diversity and the impact on epidemiological studies. Genetic alteration, if occurring *in vivo*, may explain the high diversity of *Arcobacter* present in different hosts. This variation may be caused by mutations or rearrangements in genes that are needed under stress conditions, which may increase the survival capacity of *Arcobacter*, but this hypothesis needs confirmation in future studies.

Disclosure Statement

No competing financial interests exist.

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