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# Fingerprinting and diversity of bacterial *copA* genes in response to soil types, soil organic status and copper contamination

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## Keywords

genetic diversity; copper resistance genes; soil bacterial community; genetic structure; soil organic matter; copper impact.

## Abstract

A molecular fingerprinting assay was developed to assess the diversity of *copA* genes, one of the genetic determinants involved in bacterial resistance to copper. Consensus primers of the *copA* genes were deduced from an alignment of sequences from proteobacterial strains. A PCR detection procedure was optimized for bacterial strains and allowed the description of a novel *copA* genetic determinant in *Pseudomonas fluorescens*. The *copA* DNA fingerprinting procedure was optimized for DNA directly extracted from soils differing in their physico-chemical characteristics and in their organic status (SOS). Particular *copA* genetic structures were obtained for each studied soil and a coinertia analysis with soil physico-chemical characteristics revealed the strong influence of pH, soil texture and the quality of soil organic matter. The molecular phylogeny of *copA* gene confirmed that specific *copA* genes clusters are specific for each SOS. Furthermore, this study demonstrates that this approach was sensitive to short-term responses of *copA* gene diversity to copper additions to soil samples, suggesting that community adaptation is preferentially controlled by the diversity of the innate *copA* genes rather than by the bioavailability of the metal.

## Introduction

Copper has been described for many years as an essential micronutrient for eukaryotes and prokaryotes but also as a toxic element in high concentrations, leading to detrimental effects on the metabolism of the cell (Brown *et al.*, 1992). Copper appears to be widespread in the environment, especially in agricultural soils, as a result of the application of pesticides to control plant diseases, or because of its presence as a contaminant in organic and mineral amendments (Tom-Petersen *et al.*, 2003). The combination of the significant accumulation of copper in surface soils (Flores-Vélez, 1996) and its toxicity to living organisms can therefore lead to harmful and irreversible effects on soil biological functioning and quality (Giller *et al.*, 1998).

Numerous studies have already depicted the negative effect of copper contamination on soil microbial biomass and soil key functions like mineralization of organic carbon compounds (Giller *et al.*, 1998; Dumestre *et al.*, 1999), nitrification (Sauve *et al.*, 1999) and nitrogen fixation

(McGrath, 1994). Along these lines, modifications in the population structure and diversity of the soil microbial communities as a consequence of copper contamination have been recorded by several authors following the analysis of the total phospholipid fatty acids (PLFA; Turpeinen *et al.*, 2004) or the means of new molecular methods based on the characterization of nucleic acids directly extracted from soil (Smit *et al.*, 1997; Tom-Petersen *et al.*, 2003; Ranjard *et al.*, 2006). In all cases, the impact of copper on the microbial communities found in different experiments remains difficult to compare because most studies have been limited to one soil type, without taking into account that copper bioavailability is greatly influenced by soil characteristics (Tom-Petersen *et al.*, 2003). In other respects, several authors have put forward the hypothesis that the innate composition and diversity of the indigenous community might influence its sensitivity/resistance to copper contamination and therefore partly control copper impact (Girvan *et al.*, 2004; Ranjard *et al.*, 2006). From an ecological point of view, the resistance of a community is generally described as

the static component of its stability and can result from a greater or lesser capacity to adapt to a given perturbation (Holling, 1973). At the community level, microbial adaptation is generally expressed as an increase in adapted (i.e. metal-resistant) populations and a decrease in sensitive ones (Duxbury & Bicknell, 1983; Doelman *et al.*, 1994; Ranjard *et al.*, 1997). Such an adaptation leads to a new population and functional structure (Tom-Petersen *et al.*, 2003; Ranjard *et al.*, 2006) specific to the adapted community (Baath *et al.*, 1998) which is better suited to survive in the new surrounding conditions.

*Pseudomonas syringae*, *Escherichia coli* and *Xanthomonas campestris* bacteria were the first populations reported to survive in the presence of high copper concentrations (Cooksey, 1994). Such an efficient resistance to copper resulted from the presence of additional genetic determinants organized in operons, the *cop* or *pco* operons (Cooksey, 1994). These operons are genetically homologous and encode a copper resistance mechanism that may involve copper sequestration and/or enzymatic transformation. (Cooksey, 1994; Rensing & Grass, 2003). More recently, several bacterial strains such as *Cupriavidus metallidurans* CH34 (Borremans *et al.*, 2001), *Ralstonia solanacearum* (Salanoubat *et al.*, 2002), *Xylella fastidiosa* (Simpson *et al.*, 2000), *X. campestris* (da Silva *et al.*, 2002), *Xanthomonas axonopodis* pv. *citri* str. 306 (da Silva *et al.*, 2002), *P. syringae* (Mellano & Cooksey, 1988), *Pseudomonas aeruginosa* PAO1 (Stover *et al.*, 2000), *Aeromonas veronii* bv. *sobria* (Francki *et al.*, 2000) and *Caulobacter crescentus* (Nierman *et al.*, 2001) have been shown to be resistant to copper and to possess all or part of these operons. A compilation of data available in the literature and in the international genetic databases highlights that the copper resistance of these strains has always implied the presence of *copA* homologous genes encoding a periplasmic inducible multi-copper oxidase (Nies, 1999; Rensing & Grass, 2003). Consequently, this genetic determinant may represent a relevant marker to resolve the genetic structure and diversity of the copper-resistant functional community.

The main objective of this study was to evaluate the heterogeneity of bacterial *copA* genes harboured by bacterial communities in different soils, in soils receiving different organic amendments, or in soils artificially contaminated by copper. We designed consensus PCR primers targeting *copA* genes in *Proteobacteria* and tested these primers for specificity and coverage before they were used for DNA fingerprinting of community DNA.

## Materials and methods

### Reference strains and *copA* sequences

*copA* gene sequences belonging to *Proteobacteria* and demonstrated as functionally involved in copper resistance were selected from the GenBank database (Table 1). *copA* nucleotide sequences were aligned using CLUSTALX (Thompson *et al.*, 1997) to identify conserved regions and design consensus primers (Table 2). *copA* PCR primers were tested on bacterial reference strains (Table 3). Bacteria were cultured in Luria–Bertani liquid medium at 28 °C for 24 h.

### Soil sampling

Five vineyard soils were used in this study. Auvillars, Chinon and Mâcon NA represented soil types differing in their pedologic characteristics (texture, C<sub>org</sub>, pH; Table 4). Mâcon NA, S and CC represented the same mineral matrix differing in their soil organic status (SOS) in terms of quantity and quality of SOM (Table 4). These latter soils were obtained from a long-term soil organic management (15 years old) resulting from repeated amendments with straw (S), conifer compost (CC) or not amended (NA) (Lejon *et al.*, 2007).

### Soil incubation experiment

Soil samples (150 g dry weight, sieved at 4 mm) were incubated in 2 L glass jars hermetically sealed. Soils were contaminated with CuCl<sub>2</sub> to reach a final concentration of

**Table 1.** *copA* gene sequences used for the design of PCR primers

Organisms	Accession No.	References
<i>Cupriavidus metallidurans</i> CH34	10047060	Borremans <i>et al.</i> (2001)
<i>Xylella fastidiosa</i> 9a5c	12057211	Simpson <i>et al.</i> (2000)
<i>Xanthomonas campestris</i> pv. <i>campestris</i> str. ATCC 33913	21111566	da Silva <i>et al.</i> (2002)
<i>Xanthomonas axonopodis</i> pv. <i>citri</i> str. 306	21110006	da Silva <i>et al.</i> (2002)
<i>Xanthomonas campestris juglandis</i>	349160	Lee <i>et al.</i> (1994)
<i>Pseudomonas syringae</i> plasmid pPaCu1	8272380	GenBank submission by Nakajima, 1999
<i>Escherichia coli</i> pRJ1004	619126	Brown <i>et al.</i> (1992)
<i>Pseudomonas syringae</i> pv. <i>tomato</i>	151187	Mellano & Cooksey (1988)
<i>Pseudomonas aeruginosa</i> PAO1	110227054	Stover <i>et al.</i> (2000)
<i>Ralstonia solanacearum</i> GMI1000	30407128	Salanoubat <i>et al.</i> (2002)
<i>Aeromonas veronii</i> bv. <i>Sobria</i>	6941899	Francki <i>et al.</i> (2000)
<i>Caulobacter crescentus</i>	1342236	Nierman <i>et al.</i> (2001)

**Table 2.** Alignment of *copA* sequences from the GenBank database and the forward and reverse deduced degenerated primers

Organisms	Sequences	Primer locations (pb)
<i>Cupriavidus metallidurans</i> CH34 ( <b>gi_10047060</b> )	TCGAACAAACC GGCAGCTACTGGTATCACTCGCA	456
<i>Xylella fastidiosa</i> 9a5c ( <b>gi_12057211</b> )	TACACCAATCT GGGACCTACTGGTACCACAGCCAC	444
<i>Xanthomonas campestris</i> pv. <i>campestris</i> str. ATCC 33913 ( <b>gi_21111566</b> )	TGCGCCAATCC GGCACCTACTGGTA TCACAGCCAT	429
<i>Xanthomonas axonopodis</i> pv. <i>citri</i> str. 306 ( <b>gi_21110006</b> )	TGCGCCAGTCC GGCACCTACTGGTACCACAGCCAT	438
<i>Xanthomonas campestris juglandis</i> ( <b>gi_349160</b> )	TCCAGCAGGGT GGCACCTACTGGTATCACAGTCAT	459
<i>Pseudomonas syringae</i> plasmid pPaCu1 ( <b>gi_8272380</b> )	TCAAGCAGAATGGCAC TTAAGGTTACCACAGCCAT	408
<i>Escherichia coli</i> pRJ1004 ( <b>gi_619126</b> )	TTAAGCAGAACGGGACTTACTGGTACCACAGCCAT	527
<i>Pseudomonas syringae</i> pv. <i>tomato</i> ( <b>gi_151187</b> )	TCCAACAGAACGGGACTTACTGGTACCACAGCCAC	408
<i>Pseudomonas aeruginosa</i> PAO1 ( <b>gi_110227054</b> )	TCCGGCAGAACGGCACCTACTGGTACCACAGCCAC	405
<i>Ralstonia solanacearum</i> GMI1000 ( <b>gi_30407128</b> )	TCCGGCAGAGCGGCAGCTACTGGTATCACTCGCAC	448
<i>Aeromonas veronii</i> bv. <i>sobria</i> ( <b>gi_6941899</b> )	TGGAGCAGTCT GGCACCTACTGGTA TCACTCTCAT	432
<i>Caulobacter crescentus</i> ( <b>gi_13422236</b> )	TCCGTCAGTCG GGCACCTACTGGTGGCACAGCCAT	426
Deduced degenerated primer: Coprun F2	5'-GG(GC)A(GC)(GCT)TACTGGT(AG)(GCT)CAC-3'	
	5'-GG SA SBTACTGGTRBCAC-3'	
<i>Cupriavidus metallidurans</i> CH34 ( <b>gi_10047060</b> )	TCTGCAC AACGACACGATGATGACTCACCC	1643
<i>Xylella fastidiosa</i> 9a5c ( <b>gi_12057211</b> )	CTTGGTG AATGACACCATGATGGCGCACCC	1631
<i>Xanthomonas campestris</i> pv. <i>campestris</i> str. ATCC 33913 ( <b>gi_21111566</b> )	GCTGGTC AACGACACCATGATGCAGCACCC	1604
<i>Xanthomonas axonopodis</i> pv. <i>citri</i> str. 306 ( <b>gi_21110006</b> )	GCTGGTC AACGACACCATGATGCAGCACCC	1577
<i>Xanthomonas campestris juglandis</i> ( <b>gi_349160</b> )	CCTGGTC AACGACACCATGATGTCGCACCC	1703
<i>Pseudomonas syringae</i> plasmid pPaCu1 ( <b>gi_8272380</b> )	ACTGGTA AACGACACCATGATGACCCACCC	1673
<i>Escherichia coli</i> pRJ1004 ( <b>gi_619126</b> )	GCTGATC AACGATACCATGATGACTCACCC	1732
<i>Pseudomonas syringae</i> pv. <i>tomato</i> ( <b>gi_151187</b> )	GCTGGTG AATGACACGATGATGACTCACCC	1625
<i>Pseudomonas aeruginosa</i> PAO1 ( <b>gi_110227054</b> )	CCTGGTC AACGACACCATGATGACCCACCC	1682
<i>Ralstonia solanacearum</i> GMI1000 megaplasmid ( <b>gi_30407128</b> )	CCTGCAC AACGACACGATGATGACGCACCC	1617
<i>Aeromonas veronii</i> bv. <i>sobria</i> ( <b>gi_6941899</b> )	CCTTCAT AATGATACCATGATGACACATCC	1724
<i>Caulobacter crescentus</i> ( <b>gi_13422236</b> )	GCTGATC AACGACACCATGATGCCCCATCC	1493
Deduced degenerated primer: Coprun R1	5'-TG(AGCT)G(ACT)CATCAT(GC)GT(AG)TC(AG)TT-3'	
	5'-TGNGHCATCATSGTRTCRTT-3'	

Bold type indicates nucleotides that are conserved in all 12 sequences. Regarding degenerated primer, the following one-letter code was used for variable sites: W = A or T; M = A or C; R = A or G; Y = C or T; K = G or T; S = G or C; H = A; T or C; D = G, A or T; B = G, T or C; V = G, A or C.

250 mg Cu kg<sup>-1</sup> of soil. Uncontaminated control microcosms were supplemented with pure water (H<sub>2</sub>O). The solution volume added was calculated to reach 80% of the water-holding capacity of the soil. Soil microcosms were incubated in the dark at 20 °C for 15 days, with weekly aeration and verification of soil water content.

### DNA extraction and PCR amplification of *copA* genes

Genomic DNA from bacterial strains was obtained using the Qiaquick extraction kit (Qiagen, Courtaboeuf, France). Purified DNA samples were resolved and quantified by comparison with a standard curve of calf thymus DNA (Biorad, Marnes-La-Coquette, France) by electrophoresis on a 1% agarose gel stained with ethidium bromide (Sambrook & Russel, 2001). Fragments of the *copA* gene from bacterial DNA strains were amplified by the means of degenerated oligonucleotides CoprunF2 and CoprunR1 designed in this study (Table 2). PCR reactions were conducted in a total volume of 50 µL with 5 µL of 10 × PCR buffer,

200 µM (each) deoxyribonucleoside triphosphate, 1 U of *Taq* polymerase (Qbiogene, Illkirch, France), 50 ng of DNA, 6 µM (each) primer. DNA amplification was carried out on a DNA Thermal Cycler (Eppendorf, Le Pecq, France) using the following programme: initial denaturation for 3 min at 94 °C, 35 cycles of denaturation (1 min at 94 °C), annealing (1 min at 55 °C), extension (1 min at 72 °C), and a final extension (5 min at 72 °C). PCR products were visualized through a 1.5% agarose gel electrophoresis by ethidium bromide staining.

Soil DNA was extracted from Auvillars, Chinon and Mâcon NA soils as well as from Mâcon NA, S and CC soils in contaminated and control microcosms using the method described by Ranjard *et al.* (2003). Fragments of the *copA* gene were amplified with primers CoprunF2 and CoprunR1. Hot-start PCR amplification was performed in a total volume of 25 µL with 2.5 µL of 10 × PCR buffer, 200 µM (each) deoxyribonucleoside triphosphate, 2 U of *Taq* polymerase (Qbiogene), 25 ng of soil DNA, 6 µM (each) primer, and AmpliWax bead (Applied Biosystems, CA). DNA amplification was carried out as recommended by the

**Table 3.** Bacterial strains used in this study and tested for PCR amplification with *copA* gene primers set

Strains	Phylum	Copper resistance	<i>CopA</i> detection	Sources
<i>Nocardia transvalensis</i>	Actinobacteria	Nkcr	–	Philippot L., personal data, INRA Dijon, France
<i>Rhodococcus rhodocrous</i>	Actinobacteria	Nkcr	–	Hartman A., personal data, INRA Dijon, France
<i>Bradyrhizobium japonicum</i> USDA 123	Alphaproteobacteria	Nkcr	–	Keyser <i>et al.</i> (1982)
<i>Ensifer melliloti</i> RCR 2011	Alphaproteobacteria	Nkcr	–	ATCC 51124
<i>Bradyrhizobium japonicum</i> G49	Alphaproteobacteria	Nkcr	–	IARI, Division of Microbiology, New Delhi
<i>Agrobacterium tumefaciens</i> C58	Alphaproteobacteria	Nkcr	–	ATCC 33970
<i>Cupriavidus metallidurans</i> CH34	Betaproteobacteria	+	+	Borremans <i>et al.</i> (2001)
<i>Alcaligenes faecalis</i> 8750	Betaproteobacteria	Nkcr	–	ATCC 8750
<i>Variovorax</i> sp.	Betaproteobacteria	Nkcr	–	Offre <i>et al.</i> (2007)
<i>Collimonas fungivorans</i>	Betaproteobacteria	Nkcr	–	Offre <i>et al.</i> (2007)
<i>Listeria monocytogenes</i> EGD-e	Firmicutes	Nkcr	–	ATCC BAA-679
<i>Bacillus thuringiensis</i>	Firmicutes	Nkcr	–	Cheneby <i>et al.</i> (2004)
<i>Streptomyces echinatus</i>	Firmicutes	Nkcr	–	Philippot L., personal data, INRA Dijon, France
<i>Enterococcus hirae</i>	Firmicutes	+	–	ATCC9790
<i>Staphylococcus</i> sp.	Firmicutes	+	–	Lejon <i>et al.</i> , personal data, INRA Dijon, France
<i>Paenibacillus polymyxa</i>	Firmicutes	Nkcr	–	Hartman A., personal data
<i>Listeria innocua</i>	Firmicutes	Nkcr	–	Hartman A., personal data
<i>Pseudomonas syringae</i> pv. <i>tomato</i>	Gammaproteobacteria	+	+	Mellano & Cooksey (1988)
<i>Xanthomonas campestris</i>	Gammaproteobacteria	+	+	Mellano & Cooksey (1988)
<i>Escherichia coli</i> PRJ1004	Gammaproteobacteria	+	+	Ranjard L., personal data, INRA Dijon, France
<i>Pseudomonas fluorescens</i> C7R12	Gammaproteobacteria	Nkcr	+	Eparvier <i>et al.</i> (1991)
<i>Pseudomonas putida</i> KT2440	Gammaproteobacteria	+	+	ATCC 47054

Nkcr, no known copper resistance.

**Table 4.** Physico-chemical characteristics of the soils used in this study

	Texture			pH	WHC (mL H <sub>2</sub> O 100 g soil <sup>-1</sup> )	CaCO <sub>3</sub> (g kg <sup>-1</sup> )	Organic C (g kg <sup>-1</sup> )	Organic N (g kg <sup>-1</sup> )	C/N	Cu tot (mg kg <sup>-1</sup> )	Cu EDTA (mg kg <sup>-1</sup> )
	Clay (%)	Silt (%)	Sand (%)								
Auvillars	10	29	61	7.2	25	0	10.5	0.98	11	52	28
Chinon	9	4	87	8.0	25	112	7.3	0.60	12	52	52
Mâcon NA	32	57	11	6.8	30	0	12.9	1.10	12	62	25
Mâcon S	32	58	10	6.7	35	0	19.7	1.50	13	60	27
Mâcon CC	31	56	13	6.6	40	0	45.9	2.60	18	60	22

manufacturer for Ampliwax and by temperature cycling as described above.

### ***copA* DNA fingerprinting**

The purified PCR products from soil DNA were quantified on a 1.5% agarose gel using Smart Ladder (Eurogentec, Belgium), and equal amounts of the PCR products for each sample (*c.* 500 ng) were digested at 37 °C with *HinfI* overnight. The digested PCR products were separated on a 6% acrylamide–bisacrylamide (29:1) gel for 20 h at 5 mA. The gel was then stained with SYBR Green II (Molecular Probes, Canada), scanned with a Storm 960 phosphorimager (Molecular Dynamics, Sunnyvale, CA) and analyzed using 1D-SCAN software (ScienceTec, Courtaboeuf, France). The software converted fluorescence data into electrophoretograms where peaks represented DNA fragments. The peak height was calculated in conjunction with the median filter

option and the Gaussian integration in 1D-Scan, and represented the relative proportion of the fragments in the total PCR products. Lengths (in base pairs) were calculated using a size reference mixture consisting of DNA-fragments ranging from 100 to 2000 bp. The standard was made by PCR amplifications of different fragment sizes of phage M13 mp18 (Promega, Charbonnières, France).

### ***copA* clone libraries**

Clone libraries were constructed with *copA* PCR products generated from NA, S and CC soil microcosms treated with H<sub>2</sub>O. PCR products were ligated into pGEM-T Easy according to the manufacturer's instructions (Promega). Ligated products were transformed into competent *E. coli* DH5 $\alpha$  cells following the heat shock protocol described in the manufacturer's technical manual. Transformed cells were incubated 1 h at 37 °C on a rotary shaker at 200 r.p.m. before

**Table 5.** Amounts and characteristics of humic substances from Mâcon NA, S and CC soils (from Lejon *et al.*, 2007)

	Humic substances mgC g <sup>-1</sup> soil*	Humic acid mgC g <sup>-1</sup> soil†	Fulvic acid mgC g <sup>-1</sup> soil†	β-humus mgC g <sup>-1</sup> soil†	HA/FA ratio
NA	1.70 <sup>a</sup> ± 0.20 (12.7%)	0.60 <sup>a</sup> ± 0.06 (35.3%)	0.60 <sup>a</sup> ± 0.03 (35.3%)	0.50 <sup>a</sup> ± 0.08 (29.4%)	1.00 <sup>a</sup>
S	3.50 <sup>a</sup> ± 0.49 (17.5%)	1.00 <sup>a</sup> ± 0.10 (29.0%)	1.85 <sup>b</sup> ± 0.09 (52.9%)	0.60 <sup>a</sup> ± 0.09 (17.1%)	0.55 <sup>b</sup>
CC	9.10 <sup>b</sup> ± 1.20 (25.1%)	5.0 <sup>b</sup> ± 0.50 (54.9%)	2.90 <sup>c</sup> ± 0.15 (31.9%)	1.20 <sup>b</sup> ± 0.18 (13.2%)	1.72 <sup>c</sup>

Values are means ± SD for three independent replicates.

Different superscript letters indicate significant differences ( $P < 0.05$ ) between the organic inputs for a given experimental site.

\*Percentages in parentheses are calculated as follow: humic substances (mgC g<sup>-1</sup> soil)/soil organic C (mgC g<sup>-1</sup> soil).

†Percentages in parentheses are calculated as follow: humic acids or fulvic acid or β-humus (mgC g<sup>-1</sup> soil)/humic substances (mgC g<sup>-1</sup> soil).

plating onto LB plates containing 100 µg mL<sup>-1</sup> ampicillin, 0.5 mM IPTG and 80 µg mL<sup>-1</sup> X-Gal. White *E. coli* colonies were resuspended in 10 µL dH<sub>2</sub>O, lysed for 10 min at 95 °C and subsequently briefly centrifuged. One microlitre of supernatant was used as template in a 10 µL PCR reaction using 1 µM of T7 (5'-TAATACGACTCACTATAGGG-3') and SP6 (5'-ATTTAGGTGACACTATAG-3') promoter primers. The PCR products were analyzed by agarose gel electrophoresis and clones containing DNA fragments of expected length were selected. Fifty clones in each library were randomly chosen and sequenced by GENOME express (<http://www.genome-express.com>) with a Sangers sequencing protocol using the T7-primers.

### Sequences analysis, phylogeny inference and definition of clusters

Several *copA* gene sequences (Table 1) from the GenBank database were aligned using the CLUSTALX algorithm (Thompson *et al.*, 1997) to identify conserved regions for designing consensus primers. The partial sequences amplified and cloned from soil DNA were submitted to the ORF Finder, a graphical analysis tool (<http://www.ncbi.nlm.nih.gov/gorf/gorf.html>), to identify the adequate reading frames and to deduce amino acid sequences, which were compared with sequences found in the GenBank Database (Benson *et al.*, 2005) using the BLASTP algorithm (Altschul *et al.*, 1990). Multiple-alignments were performed using the CLUSTALX algorithm (Thompson *et al.*, 1997) on DNA and protein sequences. Distance matrices were calculated using the two-parameter model of Kimura (1980) for DNA sequences and a Poisson correction for protein sequences (Galtier *et al.*, 1996). A molecular phylogenetic tree was constructed using PHYLO\_WIN (Galtier *et al.*, 1996) with the neighbour-joining method (Saitou & Nei, 1987). Bootstrap percentages (1000 bootstrap replications) were used to test the robustness of phylogenetic relationships within the tree (Felsenstein, 1985).

### Statistical analysis

Data obtained from the 1D-SCAN software were converted into a table summarizing the band presence (i.e. peak) and intensity (i.e. height or area of peak) using the PREPRISA

program (Ranjard *et al.*, 2001). For *copA* DNA profiles, 50 peaks, a 2-bp resolution and the Gaussian peak area were used for a robust analysis of profiles. A covariance matrix (microbial communities as rows and bands as columns) was subjected to principal component analysis (PCA), which provided an ordination of bacterial communities in a factorial map based on the scores of the first two principal components. Statistical ellipses representing 90% confidence were drawn over the replicates of soil samples. The Monte Carlo test with 1000 permutations was used to evaluate the significance of the discrimination revealed by PCA.

A coinertia analysis (Doledec *et al.*, 1999; Fromin *et al.*, 2002; Lejon *et al.*, 2007) was used to study the relationships between the *copA* genetic structure and the set of either soil characteristics (Table 4) or SOM characteristics (Table 5). The coupling of the soil characteristics correlation matrix and the *copA* covariance data matrix produced new PC1 and PC2 coinertia axes by projecting variables and sampling plots in a new factorial map. A randomization test of 1000 permutations was carried out to assess the statistical significance of the costructure (Monte Carlo test). When the costructure between *copA* and soil characteristics was significant, the corresponding correlation circle of soil characteristics was drawn and superposed on the *copA* factorial map to highlight the contribution of the soil parameters to the distinction of the *copA* genetic structure among the different soils.

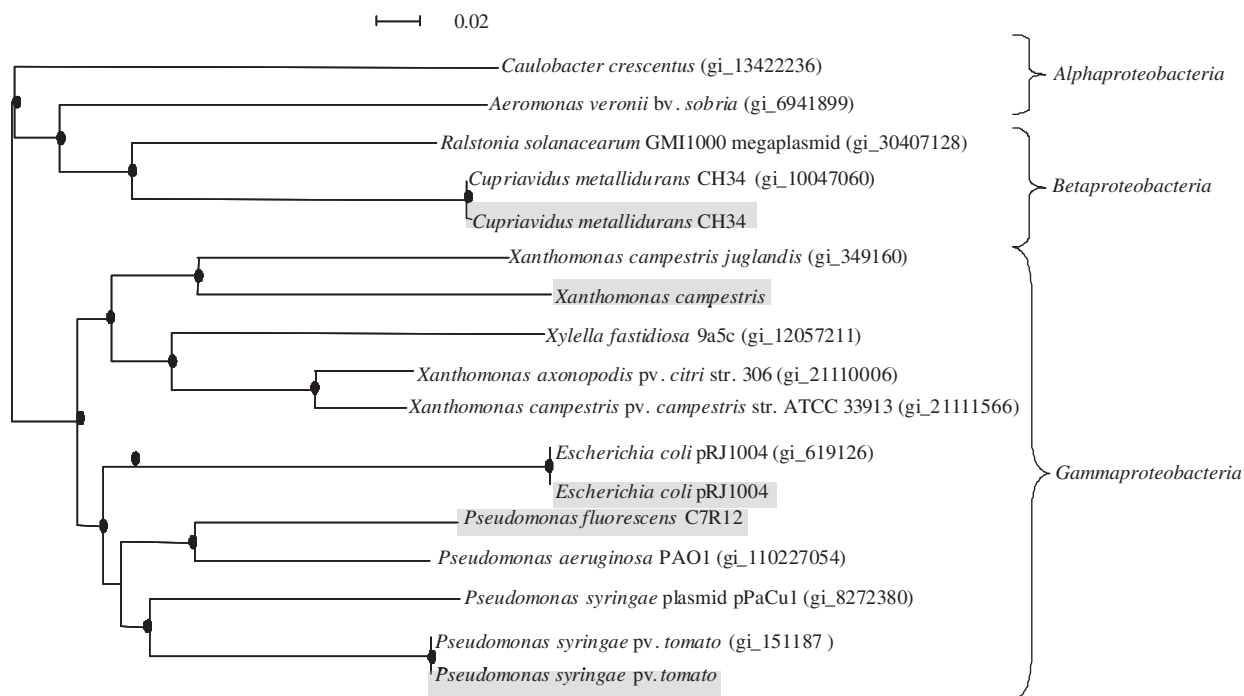
A factorial correspondence analysis (FCA) was applied to study the distribution of *copA* diversity among the different soils (NA, S and CC) by building a covariance data matrix with the frequency distribution of each cluster in each soil. The corresponding correlation circle was drawn, and arrows highlighted the relative contribution of each cluster to soil discrimination.

PCA, coinertia analysis, FCA and the Monte Carlo test were performed using ADE-4 software (Thioulouse *et al.*, 1997).

## Results and discussion

### Optimization of *copA* PCR detection and specificity

Consensus primers for the *copA* genes were deduced from an alignment of 12 *copA* sequences from proteobacterial strains



**Fig. 1.** Neighbour-joining phylogenetic tree representing *copA* nucleotide sequences from reference strains. Bootstrap values were estimated from 1000 replicates. Nineteen sequences were aligned. Horizontal distances are proportional to observed divergences expressed in substitutions per 100 sites. Bootstrap values above 80% are indicated with a black spot. Shading indicates sequences obtained after PCR amplification of *copA* gene from pure culture. Non-shaded text indicates sequences obtained from databases.

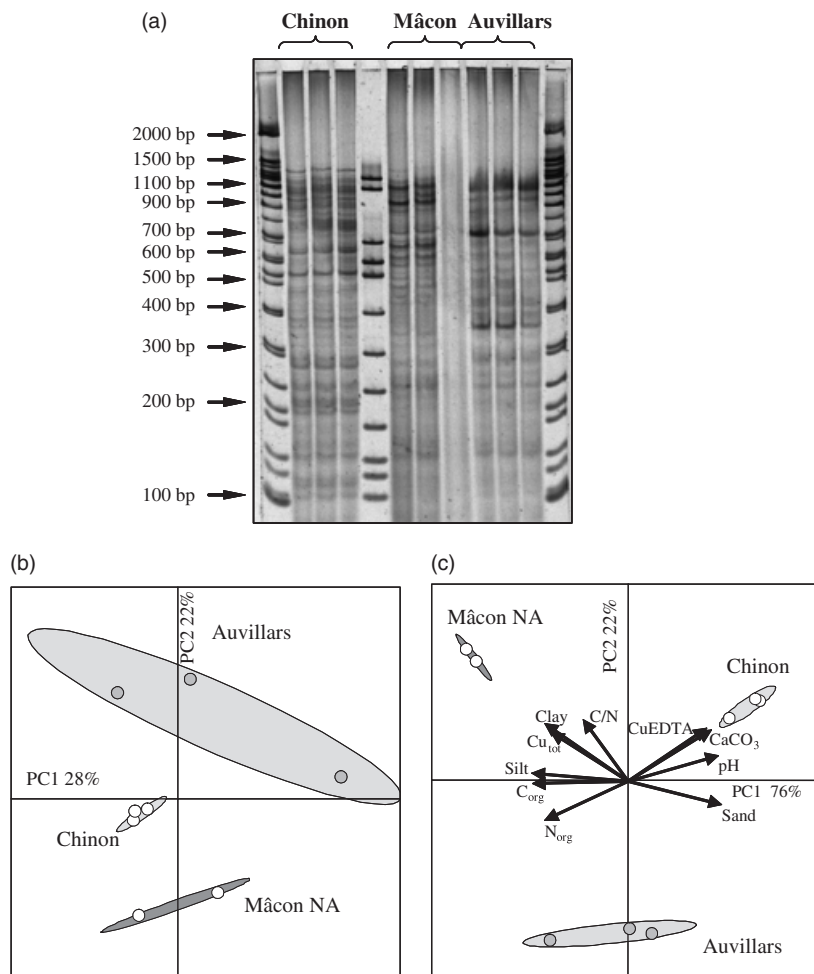
demonstrated as being copper resistant and available in the GenBank database (Table 1). The alignment of *copA* nucleotide sequences revealed only two conserved regions useful for designing degenerated forward and reverse primers: CoprunR1 and CoprunF2 (Table 2). Furthermore, a size polymorphism of this zone was recorded among determinants that ranged from 1067 bp for *Caul. crescentus* to 1277 bp for *P. aeruginosa* PAO1 (Table 2). This polymorphism emphasized the high diversity of this genetic determinant, probably due to its ancestral origin and long-term divergence between organisms (Cooksey, 1994; Nies, 1999). To obtain a maximal sensitivity for *copA* amplification from bacterial strains and soil DNA, PCR amplification was optimized by testing various hybridization temperatures, number of PCR cycles, types of *Taq* DNA polymerase, *Taq* enhancer and  $MgCl_2$  and primers concentrations (data not shown). In a first step, *copA* detection specificity was tested through the PCR amplification of genomic DNA from known resistant and sensitive bacterial strains (Table 3). PCR products of the expected size (c. 1200 bp) were generated from genomic DNA of *Cupriavidus metallidurans* CH34, *Pseudomonas syringae* pv. *tomato*, *X. campestris*, *E. coli* PRJ1004 and *Pseudomonas putida* KT2440. Furthermore, a positive amplification was also obtained for *P. fluorescens* C7R12 leading to the description of a novel *copA* genetic determinant (deposited in the GenBank under

the number EF394595). In parallel, this strain was copper resistant as it was able to grow in YG-culture media supplemented with  $CuCl_2$  (2 mM) as described by Kunito *et al.* (2001) (data not shown).

The PCR-products from these strains were sequenced and a phylogenetic tree was built up incorporating the 12 reference *copA* sequences (Table 1; Fig. 1). The phylogenetic tree confirmed the similarity between expected and amplified sequences for *Cup. metallidurans* CH34, *P. syringae* pv. *tomato*, *X. campestris* and *E. coli* PRJ1004, thus demonstrating the specificity of the designed primers. This tree also evidenced the clustering of *copA* sequences from *P. fluorescens* C7R12 with the other *copA* from the *Pseudomonas* group.

### Analysis of *copA* genetic structure in soils of variable physico-chemical characteristics

A *copA* DNA fingerprinting procedure was tested directly on soil. Total DNA from three different soils (Auvillars, Chinon and Mâcon NA; Table 4) was submitted to *copA* gene PCR amplification and to DNA fingerprinting using the restriction enzyme *Hinf*I. This enzyme was chosen by testing various restriction enzymes *in silico* (data not shown) to obtain the best discrimination between the different *copA* sequences (Table 1).

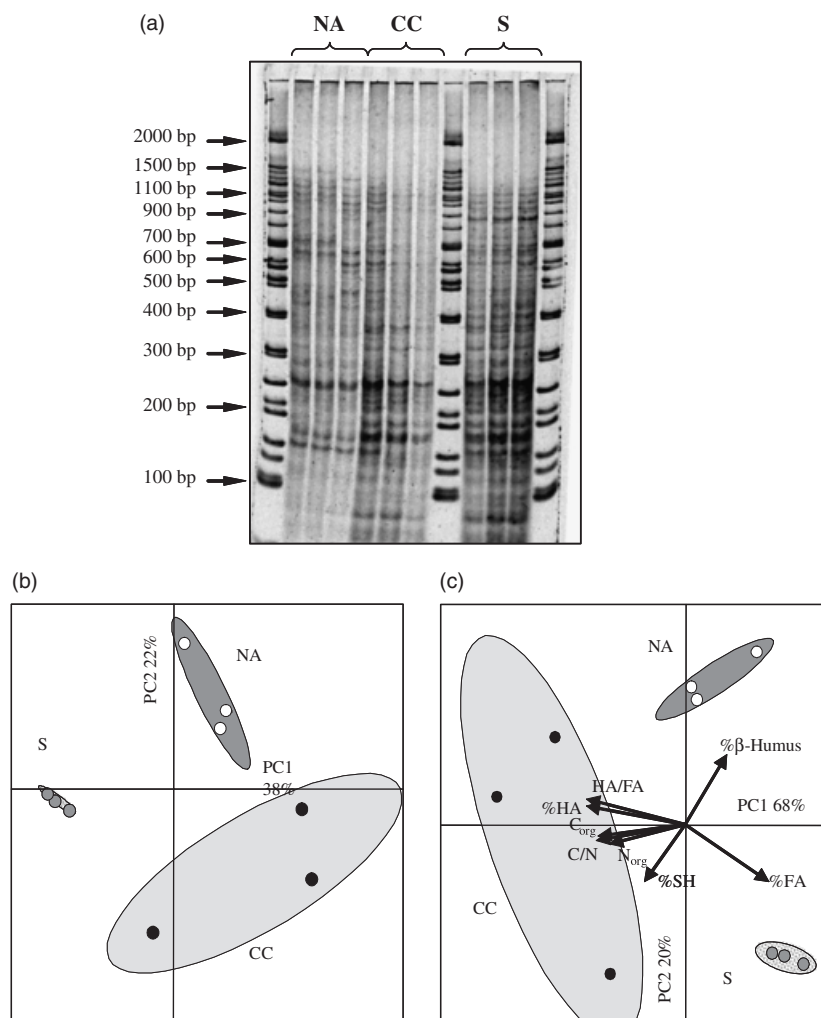


**Fig. 2.** *copA* DNA fingerprinting gel (a), principal component (PC1  $\times$  PC2) factorial map (b) generated from profiles obtained for the three different soils (Auvillars, Chinon, and Mâcon NA). Coinertia (PC1  $\times$  PC2) factorial map (c) generated from profiles and physico-chemical characteristics correlation matrices obtained for the three different soils (Auvillars, Chinon, and Mâcon NA). Arrows represent the contribution of each physico-chemical characteristic to the discrimination of the *copA* genetic structure indigenous to the different soils.

Fingerprinting of the *copA* determinants by electrophoretic separation provided complex profiles with bands ranging from 100 bp to about 1300 bp for Chinon, Mâcon NA and Auvillars soils (Fig. 2a). Such an observation is in agreement with the high diversity found among *copA* genetic determinants, as discussed above (Brown *et al.*, 1992; Cooksey, 1994). In another respect, our results also indicated a high diversity of genes encoding for copper adaptation in pristine or weakly contaminated soils such as Auvillars and Mâcon. However, the distribution of metals in the environmental matrix is known to be heterogeneous, with some micro-niches having high concentrations that may maintain a selection of resistant organisms (Ranjard *et al.*, 2000). On the other hand, as hypothesized by Bruce *et al.* (1995), a high diversity of genes encoding for metal adaptation in pristine soil can also result from (i) the close association of these genes with other genes involved in the metabolism and/or the fitness of bacteria in such an environment, and/or (ii) their role in cellular functions other than copper resistance (metal homeostasis etc.). PCA confirmed the significant discrimination between soils, essentially on the second axis,

explaining 22% of the variability (Fig. 2b). Our results suggest that different soil physico-chemical characteristics might induce a diversity of *copA* determinant. A coinertia analysis was performed to better evaluate the influence of soil characteristics on the *copA* genetic structure (Fig. 2c). The Monte Carlo tests revealed a weak but significant costructure between the *copA* genetic structure and the soil characteristics ( $P < 0.25$ ). The new factorial map deduced from the coinertia analysis of the costructure, confirmed that the *copA* genetic structure of Chinon soil was significantly discriminated from Auvillars and Mâcon NA soils on the PC1 coinertia axes, which explained 76% of the variability (Fig. 2c). Superposition of the corresponding correlation circle of physico-chemical characteristics to the PCA factorial map revealed that the particular genetic structure of Chinon was mainly explained by its texture (sandy soil) as well as its higher pH. Our observation is in agreement with recent studies depicting pH as one of the soil characteristics that have the greatest influence on bacterial diversity in soil (Fierer & Jackson, 2006). Coinertia analysis also revealed that the particular genetic structure of Chinon soil could





**Fig. 3.** *copA* DNA fingerprinting gel (a), principal component (PC1 × PC2) factorial map (b) generated from *copA* profiles of Mâcon NA, S and CC soils. Coinertia (PC1 × PC2) factorial map (c) generated from *copA* profiles covariance matrix and SOM characteristics correlation matrix obtained for Mâcon NA, S and CC soils. Arrows represent the contribution of each SOM characteristic to the discrimination of the *copA* genetic structure.

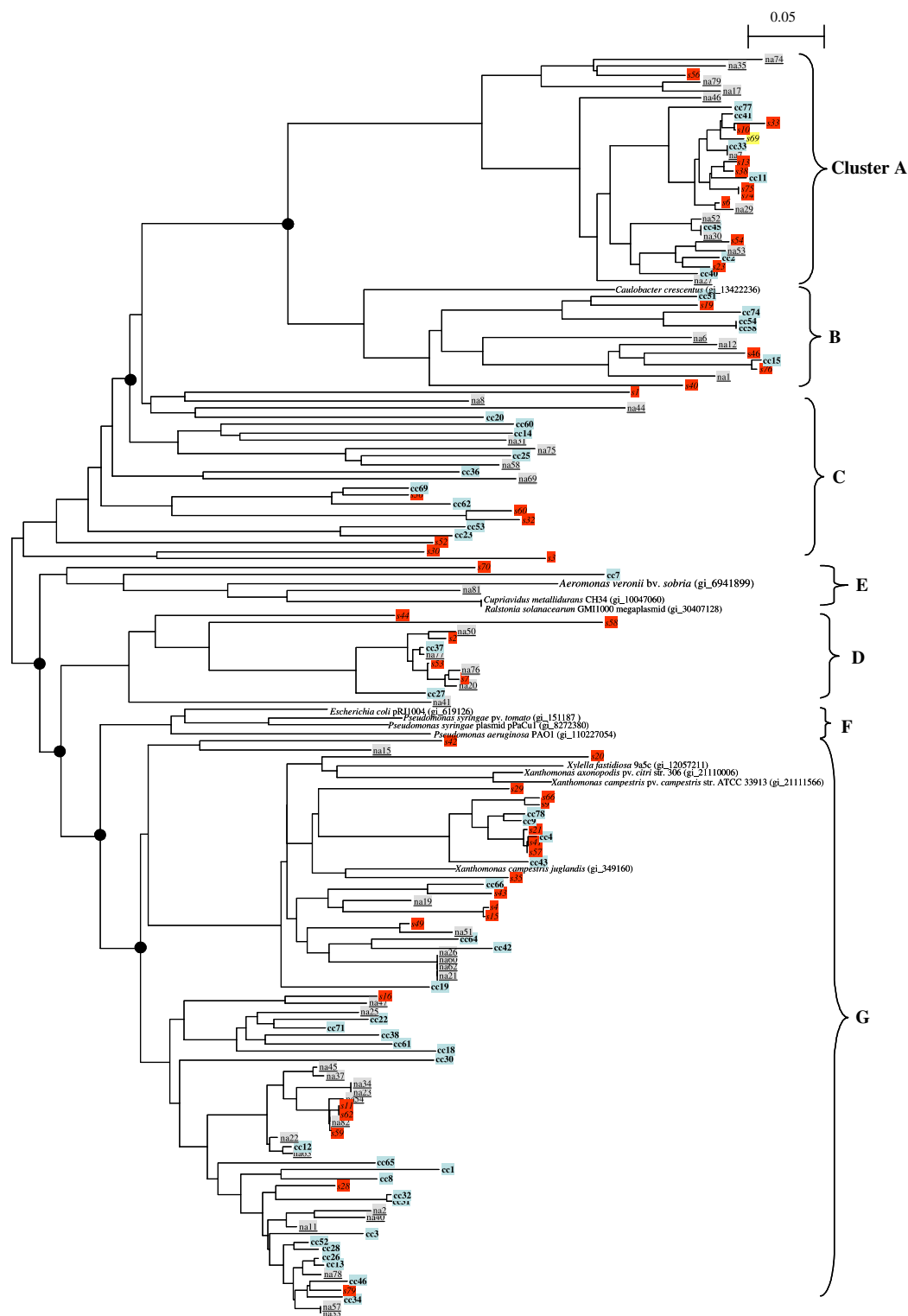
also be partly explained by a higher copper extractability, leading generally to a higher copper toxicity (Huysman *et al.*, 1994), as 52 mg Cu kg<sup>-1</sup> soil of EDTA-extractable copper were measured compared to about 25 mg Cu kg<sup>-1</sup> soil for Mâcon NA and Auvillars soils (Fig. 2c; Table 4).

### Analysis of *copA* genetic structure and diversity in soils of different organic status

The soil organic status, i.e. the type and content of soil organic matter, is well known to strongly influence the density, the activity and also the diversity of indigenous bacterial communities (Marschner *et al.*, 2003; Lejon *et al.*, 2005, 2007). In view of this, we characterized the *copA* genetic structure and diversity in the three Mâcon soils (NA, S and CC) exhibiting the same mineral matrix but different SOS, as previously described [Table 5, taken from Lejon *et al.*, (2007)]. In fact, electrophoretic profiles obtained from these three soils revealed that each SOS provided a

particular *copA* pattern (Fig. 3a), as confirmed by the PCA factorial map (Fig. 3b). NA, S and CC soils were discriminated both on the first and the second axis, which explained, respectively, 38% and 22% of the total variability.

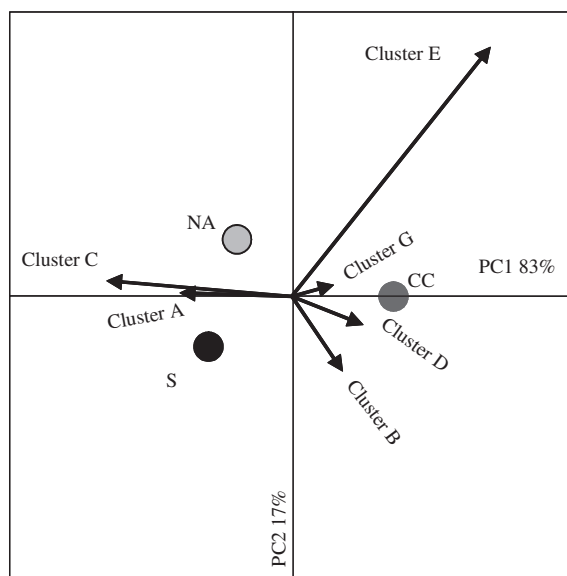
A coinertia analysis was performed using the *copA* PCA data and the SOM characteristics (Table 5). The Monte Carlo test revealed a highly significant costructure ( $P < 0.007$ ), suggesting that the SOS had a strong influence on the *copA* genetic structure. The new factorial map deduced from the coinertia analysis of the costructure confirmed that the *copA* genetic structure of the CC soil was discriminated from the NA and S soils on the PC1 coinertia axes, which explained 68% of the variability (Fig. 3c). The superposition of the corresponding correlation circle of SOM characteristics onto the *copA* factorial map revealed that the discrimination of the CC soil was mainly explained by a higher amount of SOM (higher C<sub>org</sub> and N<sub>org</sub> contents) as well as by SOM quality in terms of C/N ratio and higher content of humic acids [higher C/N and HA



**Fig. 4.** NJ phylogenetic tree of CopA amino acids sequences obtained from Mâcon (NA), Mâcon (S) and Mâcon (CC) plus reference sequences listed in Table 1; 147 sequences were aligned. Clones from Mâcon NA, S and CC were emphasized in grey, red and blue, respectively. Horizontal distances are proportional to observed divergences expressed in substitutions per 100 sites. Bootstrap values were estimated from 1000 replicates. Clusters (A–G) were defined as closely related sequences grouped together in the tree with a bootstrap > 50%. Accession number ranged from EF394448 to EF394496 for NA, from EF394497 to EF394543 for S and from EF394544 to EF394594 for CC.

(humic acid)/FA (fulvic acid) ratios] (Fig. 3c). This observation was consistent with Marschner *et al.* (2003), who reported that the quality of the SOM, as reflected by the concentration and the aromaticity of the dissolved organic carbon (DOC), affected microbial community composition. Furthermore, in a previous study, we demonstrated an influence of the SOM on the genetic structure of the dominant indigenous bacterial community [using B-ARISA fingerprinting approach (Lejon *et al.*, 2007)]. By analogy, it could be hypothesized that the modifications observed for *copA* possessing bacterial communities may be due to a lower availability and quality of the carbon source supplied by a more humified SOM (due to conifer compost decomposition) and especially to the content of recalcitrant compounds compared to a less humified SOM [e.g. with straw amendment (Domeizel *et al.*, 2004)]. In other respects, the discrimination observed between the NA and S soils was mainly due to differences in relative amounts of fulvic acids and  $\beta$ -humus (Fig. 3c), emphasizing that the quality of less humified SOM also influenced *copA* diversity. In addition, the *copA* genetic diversity was characterized by cloning and sequencing the *copA* amplified fragments from each soil. All the sequenced clones showed high similarity to *copA* sequences of *Proteobacteria* found in the databases, confirming the specificity of the primer set designed in this study. Multiple alignments and a molecular phylogenetic tree were made with these clones and the reference sequences used above (Table 1; Fig. 1). The topology of the inferred neighbour-joining (NJ) phylogenetic tree made it possible to distinguish between seven groups (clusters A–G). This was supported by bootstrap values > 50% (Fig. 4). Our results highlighted that few indigenous *copA* sequences were related with known sequences. For example, only three clones (NA81, S70 and CC7) were affiliated to the cluster E harbouring *copA* sequences from *Ralstonia*. No *copA* sequence was isolated from the cluster F, including sequences from the different *Pseudomonas* and *E. coli* determinants, whereas 69 clones grouped in the cluster G, including all the *copA* related to *Xanthomonas* and 12 in the cluster B corresponding to *Caulobacter*. It was difficult to establish a close relationship with bacterial host for clusters A, C and D.

The influence of SOS on *copA* diversity was confirmed by the factorial correspondence analysis, which was performed to resolve the diversity of the clones in the different soils (NA, S and CC). The factorial map and the correlation circle, presented in Fig. 5, revealed that the distribution of the clones differed between clusters according to the SOS. These results highlighted the separation of the CC soil from the NA and S soils on the first axis, which explained 83% of the variability, and the significant discrimination between the NA and S soils on the second axis, which explained 20% of the variability. The correlation circle evidenced that *copA* diversity in the CC soil was mainly



**Fig. 5.** Factorial correspondence analysis (FCA) generated from the distribution of the various identified clusters in NA, S and CC soils. Arrows were obtained by the superposition of the corresponding correlation circle and represent the contribution of each cluster to the discrimination of NA, S and CC soils.

represented by clusters B, D, E and G, and by clusters C and A in the NA and S soils (Fig. 5). Consequently, the specific *copA* genetic structure associated with the more humified SOM is confirmed by the occurrence of specific *copA* genes clusters in the CC soil.

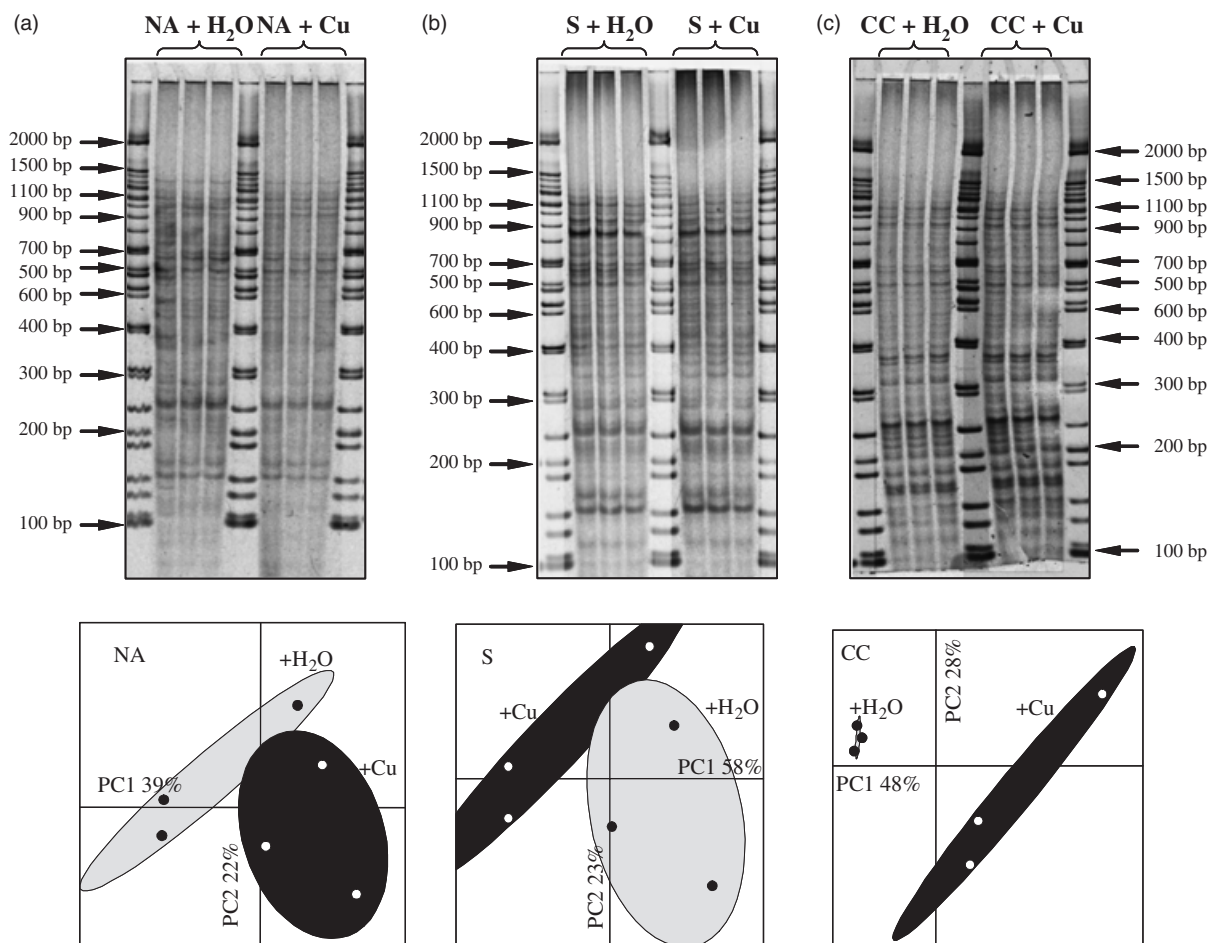
#### Analysis of community adaptation to copper contamination in soils of different SOS

The *copA* DNA fingerprinting strategy was evaluated to detect modifications consequent to artificial copper contamination. Soil incubations were carried out over 15 days with Mâcon NA, S and CC soils contaminated with copper (+CuCl<sub>2</sub> at 250 mg kg<sup>-1</sup> soil) or not (+H<sub>2</sub>O) to study short-term responses. As illustrated in Fig. 6, modifications in *copA* genetic structure were observed only in the CC soils with a significant discrimination between the control and the contaminated microcosms demonstrated by a PCA factorial map. Profiles revealed that copper spiking induced emergence of new bands and a higher intensity of pre-existing bands (shown by arrows in Fig. 6c). These results are not consistent with the hypothesis of a lower diversity of resistance genes in metal-polluted soils than in unpolluted ones (Olson *et al.*, 1989; Bruce *et al.*, 1995), possibly because of the short-term exposure described in this work. As described above, the three soils studied differed only in their SOS (quantity and type of SOM). However, our results were not consistent with the higher content of SOM in CC soil. Indeed, soil organic matter may have a major influence on

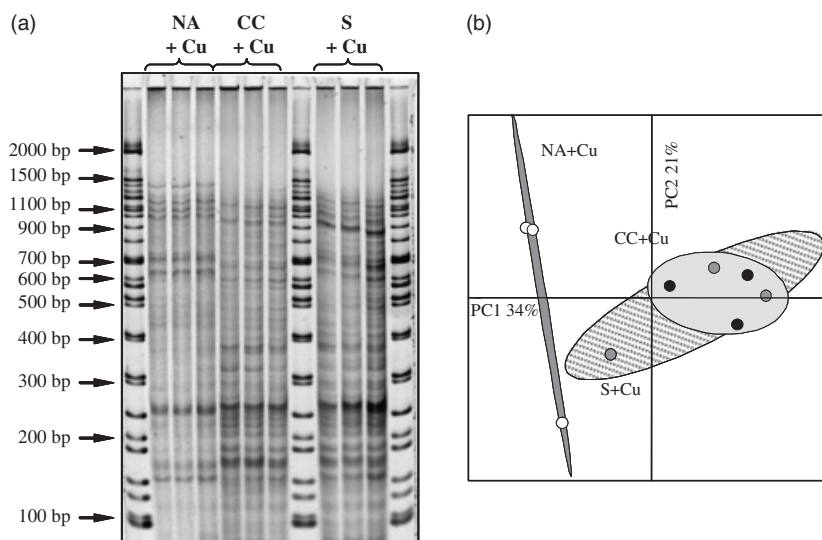
the copper impact on soil microorganisms as complexation; chelation and adsorption processes may reduce copper bioavailability and therefore toxicity (Fotovat & Naidu, 1998; Zhou & Wong, 2001; Ranjard *et al.*, 2006). In parallel, a more significant impact was recorded in the genetic structure of the dominant bacterial community of the NA compared to the S and CC soils, using B-ARISA DNA fingerprinting (unpublished data). In contrast, *copA* DNA fingerprinting results emphasized the weak relationship with total community DNA fingerprinting impact. Such a discrepancy could be explained partly by the fact that *copA* DNA fingerprinting targeted only determinants related to Proteobacteria. Consequently, this statement underlines the necessity to characterize novel *copA* determinants in Actinomycetes and Firmicutes, and to develop *copA* DNA fingerprinting specific to these taxonomic groups. On the other hand, our results might be explained partly by the fact that

bacterial copper resistance can result from other mechanisms than Cu sequestration, e.g. Cu efflux mechanisms through P-type ATPase, which are described in *E. coli* and *Enterococcus hirae* and encoded by specific genetic determinants (Magnani & Solioz, 2007).

An explanation of the higher impact observed in the CC soils might be their specific *copA* diversity as described above (see *Analysis of copA genetic structure and diversity in soils of different organic status*) (Figs 3–5). For NA and S soils, the absence of *copA* profile modifications could result from the predominance of bacterial populations harbouring highly efficient copper resistance genes that are characteristic of a highly stable genetic structure. Inversely, the significant modifications observed in the CC contaminated soil suggest the innate predominance of less efficient copper resistance genes leading to the establishment of a novel genetic structure of the community more suited to survive under



**Fig. 6.** *copA* DNA fingerprinting gels and principal component (PC1 × PC2) factorial maps generated from the profiles of soils differing in their soil organic status [Mâcon NA (a), S (b) and CC (c)] and incubated with CuCl<sub>2</sub> or with water (+H<sub>2</sub>O, control). Arrows indicate significant modifications between control and contaminated CC soils.



**Fig. 7.** *copA* DNA fingerprinting gel (a) and principal component (PC1 × PC2) factorial map (b) generated from the profiles of the Mâcon NA, S and CC soils contaminated with CuCl<sub>2</sub>.

metal stress. This hypothesis is consistent with the results illustrated by the factorial map generated from the *copA* profiles of contaminated soils (Fig. 7). These results revealed that the new *copA* genetic structure of the CC contaminated soil is similar to that of the S soil and is discriminated from the NA soil on the first axis, which explained 34% of the variability. In this context, further investigations by cloning and sequencing *copA* genes from CC and S contaminated soils could allow confirmation of such a similarity in *copA* diversity. In addition, as it is well known that copper spiking induced an enrichment of adapted populations (Duxbury & Bicknell, 1983; Huysman *et al.*, 1994; Kunito *et al.*, 1997), complementary analysis could be performed to compare the abundance of *copA* genes before and after copper contamination and to reach a more precise conclusion about the impact of copper in the different soils. Although quantitative PCR is a relevant approach to quantify functional genes in soil (Kandeler *et al.*, 2006), our primers are not adequate for this approach as they were degenerated and provided PCR fragments of different sizes that were too large to be quantified robustly.

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