International Journal of Dairy Technology SDT SOCIETY OF DAIRY TECHNOLOGY

doi: 10.1111/1471-0307.12211

SHORT COMMUNICATION

First isolation of *Brevibacterium* sp. pigments in the rind of an industrial red-smear-ripened soft cheese

PATRICK GALAUP,[1] NUTHATHAI SUTTHIWONG,[2,3] MARIE-NOËLLE LECLERCQ-PERLAT,[4] ALAIN VALLA,[5] YANIS CARO,[3] MIREILLE FOUILLAUD,[3] FABIENNE GUÉRARD[1,6] and LAURENT DUFOSSÉ[1,3]*

[1]*Laboratoire ANTiOX, Université de Bretagne Occidentale, Pôle Universitaire Pierre-Jakez Hélias, Créac'h Gwen F-29000, Quimper, France,* [2]*Agricultural Technology Department, Thailand Institute of Scientific and Technological Research, Technopolis 35 Mu 3, Klong 5, TH-12120, Pathum Thani, Thailand,* [3]*Laboratoire de Chimie des Substances Naturelles et des Sciences des Aliments, Université de La Réunion, ESIROI Agroalimentaire, Parc Technologique, F-97490 Sainte-Clotilde, Ile de La Réunion, France,* [4]*INRA UMR 782 Génie et Microbiologie des Procédés Alimentaires, F-78850 Thiverval-Grignon, France,* [5]*CNRS FRE 2125, Chimie et Biologie des Substances Naturelles, F-29000 Quimper, France, and* [6]*Université Européenne de Bretagne, Institut Universitaire Européen de la Mer, Technopôle Brest-Iroise, F-29280 Plouzané, France*

The smear-ripened soft cheeses are characterised by a surface orange-red-brown colour, which has a microbial origin. For a long time, this colouration was mainly imputed to Brevibacterium linens. *However, the latest published works, based on molecular biology, have shown a minor role for this bacterium. This study shows the results obtained with an industrial cheese named Vieux-Pané, which is characterised by the presence of carotenoids from* Brevibacterium linens *group at its surface. This demonstrates that, under certain conditions, the* Brevibacterium linens *group (*Brevibacterium linens *and* Brevibacterium aurantiacum *sp. nov.) is able to produce pigments and to colour cheeses effectively.*

Keywords *Brevibacterium linens*, carotenoid, isorenieratene, cheese rind, colour.

INTRODUCTION

A previous study from our laboratory presented an extraction method for the analysis of pigments in the cheese rinds (Galaup *et al.* 2005). This method was then applied to high-quality red-smear soft cheeses such as those under the 'Protected Designation of Origin (PDO)' legislation, and the first pigment fingerprints of such cheeses were obtained (Galaup *et al.* 2005). It has also shown that the origin of pigments seems essentially related to the presence of yellow bacteria such as *Arthrobacter* or *Microbacterium* species (Galaup *et al.* 2007), the pigments of *Brevibacterium linens* group being only present at trace amounts on Maroilles (Guyomarc'h *et al.* 2000a) (Data S1 and S2 in Supporting Information). All these studies seem to confirm the minor role of *Brevibacterium linens* group in cheese colouring.

We have recently applied this method to different industrial cheeses, and this paper shows the pigment fingerprint of the rind of Vieux-Pané produced in France, which is characterised by the predominant presence of pigments from *Brevibacterim linens* group.

MATERIALS AND METHODS

Bacterial strains, culture media, cheeses, extraction of pigments and HPLC conditions were described in previous publications (Guyomarc'h *et al.* 2000b; Galaup *et al.* 2005). (Data S3 in Supporting Information).

RESULTS AND DISCUSSION

HPLC profiles of the *Brevibacterium linens* extracts

For all strains investigated (*Brevibacterium linens* and *Brevibacterium aurantiacum* sp. nov.), the colour reaction of biomass or methanol extracts in the presence of alkali is positive. It is due to the ionisation of phenols into phenolates in both mono- and di-hydroxylated isorenieratene derivatives (Britton *et al.* 1995). This reaction is characteristic of the pigments of the *Brevibacteri-*

*Author for correspondence. E-mail: laurent.dufosse@univ-reunion.fr

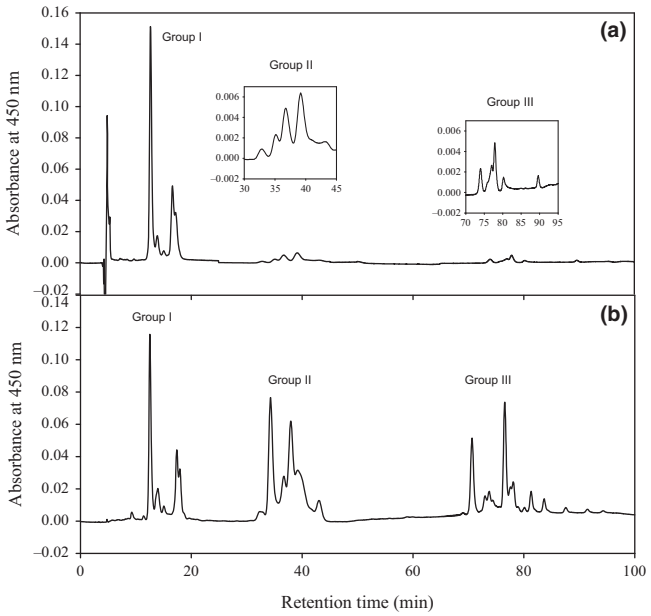

Figure 1 Chromatographic profile of pigments from *Brevibacterium aurantiacum* ATCC 9175 methanolic extract (a) and chromatographic profile of pigments extracted from the rind of Vieux-Pané red-smear soft cheese (b).

um linens group. Moreover, all strains (over one hundred strains tested up to now in all of our experiments – including the 30 strains of the present work – have shown the same typical chromatogram, even if some of these strains (e.g. ATCC 9175) are reclassified as *Brevibacterium aurantiacum* (Gavrish *et al.* 2004). Even if it is possible to differentiate these two species by molecular biology methods, they show the same pigment fingerprint and develop a carmine colour in contact with strong alkali.

The chromatographic profile of *Brevibacterium linens* or *Brevibacterium aurantiacum* (Figure 1a) consisted of a series of three groups of peaks (Guyomarc'h *et al.* 2000b). The first group (I) was eluted between 15 and 22 min. A main peak elutes between 15–17 min, followed 1.5 min later by a smaller one. Between 17 and 18 min, 2 other peaks were eluted. The second group (II) consisted of a series of small peaks eluted between 30 and 45 min. The last group of peaks (III) to be detected was eluted between 70 and 80 min. The three groups of peaks were identified as respectively related to 3,3′-dihydroxy-isorenieratene (group I), 3-hydroxy-isorenieratene (group II) and isorenieratene (group III). On a synthetic medium, *Brevibacterium linens* produced mainly the 3,3′-dihydroxy-isorenieratene. The groups representing the 3-monohydroxy and isorenieratene were present only as traces.

By the study of UV/visible spectra, more information could be obtained such as *cis*/*trans* isomerisation (Schieber and Carle 2005) or hydroxylation. The identification of the *cis* compounds was possible due to their characteristic '*cis*-peak' (320-380 nm, see Data S4 in Supporting Information). Moreover, when a compound is hydroxylated, it becomes more polar and presents a more rounded UV/visible spectrum.

The compounds, which are eluted in each group, seem to be isomers of the same molecule. Indeed, as in the case of lycopene (Chasse *et al.* 2001; Ishida *et al.* 2001), a quite high number of *cis*/*trans* isomers of isorenieratene, monohydroxy-isorenieratene and di-hydroxy-isorenieratene could be obtained, based on the position of the isomerisation (Figure 2, *cis* isomers of 3-3′-dihydroxy-isorenieratene).

HPLC profiles of the cheese extracts

The profile presented the three distinct groups of pigments very characteristic of the *Brevibacterium linens* group (Figure 1b). A first group of pigments was eluted between 12 and 18 min (group I), including dihydroxy-isorenieratene and its isomers (all-*trans* molecule and various *cis* isomers). A second group (II) was characterised by a very important set of compounds eluted between 30 and 45 min and corresponded to 3-monohydroxy-isorenieratene and its isomers. The third group consisted of some isorenieratene isomers. Since this molecule was not commercially available, the identification was made with isorenieratene chemically synthesised in our laboratory (Valla *et al.* 2007).

Comparison between the two profiles

The profiles obtained in the present study on cheeses showed a strong similarity with pigment profiles of *Brevibacterium linens* extracts (Figure 1), with three groups of pigments (groups I, II and III), of variable polarity (Guyomarc'h *et al.* 2000a). That was confirmed by the analysis of the UV/visible absorption spectra and of retention times. The similarity between the group of 3,3′-dihydroxy-isorenieratene (retention times from 12 to 18 min) present in synthetic medium and cheese matrix was clear. Even if the concentrations in pigments of groups II and III were very different among the 2 profiles, the molecules were identical. The pigments extracted from the cheese rind represented all isomers of isorenieratene and hydroxyl derivatives described in *Brevibacterium linens* by Kohl *et al.* (1983).

Pigments of the groups II and III are not generally detected at this high level of concentration in the extracts of pigments resulting from *Brevibacterium linens* cultivated in synthetic media. It was thus the first time that the three groups of pigment with such intensity and resolution were detected.

The presence of these molecules on the rind of cheeses raises some questions about their production. Was the production of these molecules induced by factors (e.g. biotic, physico-chemical. . .) that are not present or effective on synthetic culture medium? Was an interaction with other micro-organisms (e.g. yeasts, bacteria) necessary for the production of substantial amounts of mono-hydroxy-isorenieratene and isorenieratene? In the literature, the study of the production of pigments from this bacterium was primarily and mainly conducted with synthetic culture media. In the future, it would be

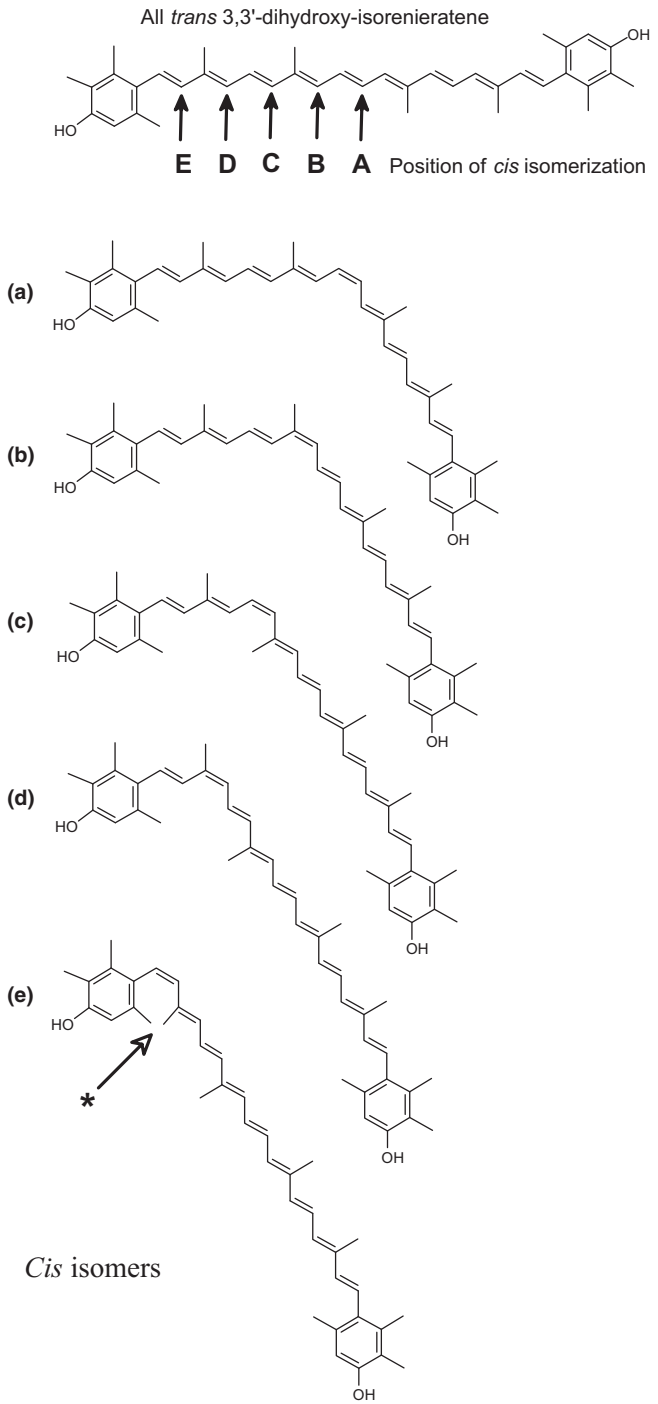

Figure 2 Isomers of 3,3′-dihydroxy-isorenieratene, concerning only one isomerisation of the ethylenic linkage (the most probable), that is the *all*-trans isomer and the five *cis* isomers that could occur. *Cis* isomers are named from A to E, starting from the middle of the molecule. *Indicates a steric hindrance. Similar isomers could be described for non-hydroxylated isorenieratene and 3-hydroxy-isorenieratene.

judicious to use complex and/or dairy media reflecting the more the composition of a cheese, such as curd models or 'cheese models' used by Leclercq-Perlat *et al.* (2004).

Latest studies concerning the microflora of red-smear-ripened soft cheeses showed a weak presence of *Brevibacterium linens* group. Bockelmann (2002) reported that the yellow pigmented *Arthrobacter nicotianae* accounts for 5–10% of the bacteria isolated from Tilsit, whereas *Brevibacterium linens* accounts for 0.1–10% (Bockelmann *et al.* 2005). In Comté, less than 1% of the total flora consists of *Brevibacterium linens* (Bockelmann *et al.* 2005). The 16S DNA sequences coding for *Brevibacterium linens* are barely detected at the end of the ripening of red-smear-ripened soft cheeses, the bacteria present being especially species of *Arthrobacter* (Feurer *et al.* 2004). In French PDO red-smear cheeses, the *Brevibacterium linens* pigment fingerprint was only slightly detected on the Maroilles cheese (Galaup *et al.* 2007). With all these various works and results, the tendency was to minimise the role of *Brevibacterium linens* in favour of other micro-organisms (Mounier *et al.* 2006; Goerges *et al.* 2008).

Since 1997, Bockelmann and his team have studied which bacterium could lead to the complete formation of cheese models close to Tilsit from a point of view of odour and colour. While the sole presence of *Brevibacterium linens* produced imperfect cheeses, the combination of *Brevibacterium linens* with *Arthrobacter* sp. led to acceptable cheeses. More complex combinations between *Debaryomyces hanseni*, *Arthrobacter* sp. (yellow strain) and *Staphylococcus* sp. (pigmented and not pigmented strains) could give colour and odour similar to those of Tilsit (Bockelmann 2002; Bockelmann *et al.* 2005). The whole of this work led to an effective cocktail of micro-organisms for the ripening of Tilsit containing five species: *Debaryomyces hansenii, Brevibacterium linens, Staphylococcus equorum (S. sciuri), Corynebacterium ammoniagenes* and *Arthrobacter nicotianae* (Bockelmann 2002; Bockelmann *et al.* 2005).

Our study, in the case of this French industrial cheese Vieux-Pané, demonstrated that a strain from *Brevibacterium linens* group was able to produce pigments and impart colour to a cheese with efficacy. The biodiversity of the bacterial flora on the surface of cheese is important (Brennan *et al.* 2002; Bokulich and Mills 2013). In this case of industrial cheeses (Feligini *et al.* 2012; Gori *et al.* 2013), the composition of the various micro-organisms seeded during the process is not known and is confidential. It may occur that *Brevibacterium linens* did not have to undergo competition with other micro-organisms and that its development at the cheese surface could happen in an optimal way.

CONCLUSIONS

The weak occurrence of *Brevibacterium linens* and *Brevibacterium aurantiacum* sp. nov. at the surface of red-smear-ripened soft cheeses has been shown in previous studies and the role of these bacteria on the development of cheese colour has been minimised. In fact, cheese rind colouration is a complex process and our results showed

that this group of bacteria, under certain conditions, was quite able to give colour to cheese surface so, the role of *Brevibacterium linens* group is not negligible.

Future research would explain why this group of bacteria (always present at the earliest stage of ripening) produced pigments on certain cheeses and not on others.

ACKNOWLEDGEMENTS

This work was supported by ACTIA (Association de coordination technique pour l'industrie agro-alimentaire) research programmes, the French Minister of National Education, Research and Technology, Degussa France and four Protected Designation of Origin cheesemakers. Patrick Galaup is grateful to Dr. Régis Moreau (Linus Pauling Institute, Corvallis, USA) for helpful discussion. Laurent Dufossé thanks the so-called Carotenoid *triumvirate*, Synnøve Liaaen-Jensen, Hanspeter Pfander and George Britton for nice discussions about carotenoids, fascinating chemical compounds. Laurent Dufossé, Yanis Caro and Mireille Fouillaud would like to thank to the Conseil Régional de La Réunion, Reunion Island, France, for financial support of research activities dedicated to microbial pigments.

REFERENCES

Bockelmann W (2002) Development of defined surface starter cultures for the ripening of smear cheeses. *International Dairy Journal* **12** 123–131.

Bockelmann W, Willems K P, Neve H and Heller K J (2005) Cultures for the ripening of smear cheeses. *International Dairy Journal* **15** 719–732.

Bokulich N A and Mills D A (2013) Facility-specific "house" microbiome drives microbial landscapes of artisan cheesemaking plants. *Applied and Environmental Microbiology* **79** 5214–5223.

Brennan N M, Ward A C, Beresford T P, Fox P F, Goodfellow M and Cogan T M (2002) Biodiversity of the bacterial flora on the surface of a smear cheese. *Applied and Environmental Microbiology* **68** 820–830.

Britton G, Liaaen-Jensen S and Pfander H (1995) Carotenoids today and challenges for the future. In *Carotenoids, Volume 1A: Isolation and Analysis*, pp 13–26. Britton G, Liaaen-Jensen S, Pfander H, eds. Basel: Birkhäuser Publ.

Chasse G A, Mak M L, Deretey E *et al.* (2001) An ab initio computational study on selected lycopene isomers. *Journal of Molecular Structure* **571** 27–37.

Feligini M, Panelli S, Buffoni J N, Bonacina C, Andrighetto C and Lombardi A (2012) Identification of microbiota present on the surface of Taleggio cheese using PCR-DGGE and RAPD-PCR. *Journal of Food Science* **77** M609–M615.

Feurer C, Vallaeys T, Corrieu G and Irlinger F (2004) Does smearing inoculum reflect the bacterial composition of the smear at the end of the ripening of a French soft, red-smear cheese? *Journal of Dairy Science* **87** 3189–3197.

Galaup P, Flamin C, Carlet E and Dufossé L (2005) HPLC analysis of the pigments produced by the microflora isolated from the 'Protected Designation of Origin' French red-smear soft cheeses Munster, Epoisses, Reblochon and Livarot. *Food Research International* **38** 855–860.

Galaup P, Gautier A, Piriou J, de Villeblanche A, Valla A and Dufossé L (2007) The first pigment fingerprints obtained from the rind of French red-smear ripened soft cheeses. *Innovative Food Science and Emerging Technologies* **8** 373–378.

Gavrish E Y, Krauzova V I, Potekhina N V, Karasev S G, Plotnikova E G, Altyntseva O V, Korosteleva L A and Evtushenko L I (2004) Three new species of brevibacteria, *Brevibacterium antiquum* sp. nov., *Brevibacterium aurantiacum* sp. nov., and *Brevibacterium permense* sp. nov. *Microbiology* **73** 176–183.

Goerges S, Mounier J, Rea M C, Gelsomino R, Heise V, Beduhn R, Cogan T M, Vancanneyt M and Scherer S (2008) Commercial ripening starter microorganisms inoculated into cheese milk do not successfully establish themselves in the resident microbial ripening consortia of a south German red smear cheese. *Applied and Environmental Microbiology* **74** 2210–2217.

Gori K, Ryssel M, Arneborg N and Jespersen L (2013) Isolation and identification of the microbiota of Danish farmhouse and industrially produced surface-ripened cheeses. *Microbial Ecology* **65** 602–615.

Guyomarc'h F, Binet A and Dufossé L (2000a) Characterization of *Brevibacterium linens* pigmentation using spectrocolorimetry. *International Journal of Food Microbiology* **57** 201–210.

Guyomarc'h F, Binet A and Dufossé L (2000b) Production of carotenoids by *Brevibacterium linens*: variation among strains, kinetic aspects and HPLC profiles. *Journal of Industrial Microbiology and Biotechnology* **24** 64–70.

Ishida B K, Ma J and Chan B (2001) A simple, rapid method for HPLC analysis of lycopene isomers. *Phytochemical Analysis* **12** 194–198.

Kohl W, Achenbach H and Reichenbach H (1983) The pigments of *Brevibacterium linens*: aromatic carotenoids. *Phytochemistry* **22** 207–210.

Leclercq-Perlat M N, Corrieu G and Spinnler H E (2004) The color of *Brevibacterium linens* depends on the yeast used for cheese deacidification. *Journal of Dairy Science* **87** 1536–1544.

Mounier J, Fitzgerald G F and Cogan T M (2006) Survival of surface ripening cultures during storage and monitoring their development on cheese. *Letters in Applied Microbiology* **42** 425–431.

Schieber A and Carle R (2005) Occurrence of carotenoid cis-isomers in food: technological, analytical, and nutritional implications. *Trends in Food Science and Technology* **16** 416–422.

Valla A, Andriamialisoa Z, Valla B, Labia R, Le Guillou R, Dufossé L and Cartier D (2007) A new biomimetic-like aromatization of the cyclic end groups of terpenoids with stereospecific migration of one of the methyl groups: a convenient route to isorenieratene (ϕ, ϕ-carotene). *European Journal of Organic Chemistry* **4** 711–715.

SUPPORTING INFORMATION

The following supporting information is available for this article:

Data S1. Smear-cheeses with rinds colored by a bacterial microflora. 1a. Livarot, 1b. Rollot in the ripening cellar, 1c. Hand-made washing of Munster, 1d. Maroilles.

Data S2. Aromatic carotenoids described in Brevibacterium linens according to Kohl *et al.* (1983).

Data S3. Material and Methods

Data S4. UV-visible spectra of 3,3′-dihydroxy-isorenieratene (a: all-trans isomer, a': cis isomer), 3-hydroxyisorenieratene (b: all-trans isomer, b': cis isomer), and isorenieratene (c: all-trans isomer, c': cis isomer). Arrows indicate the peak in the 320-380 nm range, specific for cis rearrangement.