Physical and genetic map of the genome of *Staphylococcus carnosus* TM300

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A genome map of *Staphylococcus carnosus* TM300, an important micro-organism in the food industry and long used as a starter culture, was constructed by pulsed-field gel electrophoresis of DNA fragments obtained after digestion with *NotI*, *SfiI* and *Apal*. The size of the chromosome was estimated to be 2590 kb. The fragments were assembled into a physical map using a combination of complementary methods including multiple and partial digests of genomic DNA, hybridization with homologous gene probes, and cross-Southern hybridization. Fifteen genes or gene clusters were positioned on the physical map by Southern hybridization analysis. The map provides a basis for further analysis of the *S. carnosus* chromosome.

Keywords: *Staphylococcus carnosus*, genome mapping

INTRODUCTION

It has been known for a long time that Gram-positive and catalase-positive cocci play an important role in the ripening process of dry sausages. The predominant micro-organism in fermented meat is *Staphylococcus carnosus*, referred to in the early literature as *Micrococcus* (Götz & Schleifer, 1983; Lerche & Sinell, 1955; Niinivaara & Pohja, 1956). On the basis of modern taxonomic criteria such as DNA sequence homology, chemical composition of the peptidoglycan, and biochemical properties, the organism was reclassified as a new staphylococcal species, *S. carnosus*; the species name of the bacterium is derived from its occurrence in meat (Schleifer & Fischer, 1982). Since the 1950s, *S. carnosus* has been used alone or in combination with other micro-organisms, such as pediococci or lactobacilli, as a starter culture for the production of raw sausage. The main positive characteristics of *S. carnosus* in this process are its ability to reduce nitrate, to develop a characteristic flavour, to lower the pH moderately, and to reduce hydrogen peroxide produced by the catalase-negative lactobacilli (Liepe, 1982).

S. carnosus has only a low DNA sequence homology with *S. aureus* and other staphylococcal species. It produces no toxins, haemolysins, protein A, coagulase, or clumping factors, markers which are typical for many *S. aureus* strains. As *S. carnosus* is such a commercially useful micro-organism, we thought it particularly worthwhile to develop a gene cloning system for it (Götz, 1986, 1990a, b). Among the various *S. carnosus* strains

we chose *S. carnosus* strain TM300 because of its low extracellular proteolytic activity. Transformation systems have been worked out for this strain (Augustin & Götz, 1990; Götz *et al.*, 1983; Götz & Schumacher, 1987) and suitable cloning vectors have been developed (Kreutz & Götz, 1984; Rosenstein *et al.*, 1992; Wieland *et al.*, 1995). *S. carnosus* TM300 is used as an alternative host organism not only in molecular genetics to study staphylococcal-related pathways (Augustin *et al.*, 1992; Ayora & Götz, 1994; Heilmann *et al.*, 1996; Neubauer & Götz, 1996; Wieland *et al.*, 1994) but also to produce and secrete heterologous proteins or to display the proteins on the cell surface (Demleitner & Götz, 1994; Liebl & Götz, 1986; Samuelson *et al.*, 1995; Strauss & Götz, 1996; Thumm & Götz, 1997).

Despite the establishment of a host vector system and the availability of many cloned genes, the structure and organization of the *S. carnosus* genome is not well defined.

Recently the genomes of 47 coagulase-negative staphylococcal strains (CNSs) assigned to different species were analysed by pulsed-field gel electrophoresis (PFGE) (Snopková *et al.*, 1994). The strains were clustered on the basis of their similarity in the *SmaI* restriction patterns into various groups, which fitted well with the classification scheme of CNSs. It was also demonstrated that by comparison of genome restriction patterns, so far uncharacterized CNSs could be allocated to certain species.

PFGE (Schwartz & Cantor, 1984) has proved to be an

efficient method for genome size estimation and construction of chromosomal maps as well as being a useful tool for the epidemiological analysis of bacterial species. The construction of physical maps has provided much information on the number and topology of chromosomes, the location of genes, and the genomic flexibility and structural conservation of a given species by comparative mapping of stains. Genome mapping might be considered out of fashion in the days of genome sequencing projects. However, a thoroughly worked out genomic map, such as that of *S. aureus* (Pattee *et al.*, 1990), is an extremely valuable basis for starting genome sequencing.

Here we present a physical map of the chromosome of *S. carnosus*. The map was constructed by PFGE of fragments generated by restriction enzymes that cut rarely, in combination with the hybridization of specific gene probes. The generation of a physical map for this organism will help direct future identification, gene cloning, fine mapping strategies and genome sequencing.

METHODS

Preparation of DNA for PFGE. DNA was prepared in agarose blocks by previously described methods (Linhardt *et al.*, 1992; Prévost *et al.*, 1992) with some modifications. *S. carnosus* TM300 was grown in 20 ml 2 × YT medium at 37 °C to an OD₅₇₈ of 0.2. After addition of 200 µl 0.5 M EDTA (pH 8.0) the bacteria were harvested, washed twice in 2 ml of a buffer containing 10 mM Tris/HCl (pH 7.5), 10 mM EDTA, 10 mM EGTA and 1 M NaCl, resuspended in 200 µl of the same buffer, and stored at 55 °C. A 7–10 µl volume of a lysostaphin (Sigma) stock solution (0.5 mg ml⁻¹) was added to 100 µl of the suspension, which was then immediately mixed with 100 µl 2% low-melting-point agarose (BRL) in 50 mM Tris/HCl (pH 8.0), 5 mM EDTA. The mixture was cast in moulds to form agarose blocks, which were subsequently transferred into 1 ml lysis buffer [6 mM Tris/HCl (pH 7.6), 100 mM EDTA, 1 M NaCl, 0.5% Brij 58, 0.2% sodium deoxycholate, 0.5% *N*-lauroylsarcosine, pH 7.6] supplemented with 500 µg lysozyme (Boehringer Mannheim) and incubated for 2–3 h at 37 °C. The lysis buffer was then replaced with 1 ml proteolysis buffer [0.25 M EDTA, 20 mM EGTA, 1% (v/v) *N*-lauroylsarcosine] containing 500 µg proteinase K (Boehringer Mannheim). Digestion was performed for 12 h at 55 °C. The agarose blocks were washed five times with 10 ml TE buffer and stored at 4 °C in TE buffer.

Endonuclease digestion and PFGE. Digestions of the high-molecular-mass DNA were performed with different restriction endonucleases purchased from Boehringer Mannheim (*Not*I, *Sfi*I, *Apa*I, *Ecl*XI, *Ksp*I, *Bss*HII), New England Biolabs (*Asc*I), Amersham (*Sse*8387I), BRL (*Sma*I) and Promega (*Sgf*I). The agarose blocks were cut into pieces (2 × 1 × 4 mm), which were incubated with 10–20 U of the enzyme in a final volume of 100 µl for 2–4 h at the required reaction temperature. Multiple digestions were done sequentially by washing the agarose pieces and changing the buffer to the appropriate one between the incubations. Some double digestions were analysed by two-dimensional PFGE. After digestion with one enzyme and electrophoresis in one dimension, DNA fragments contained in excised gel blocks were digested with 50–200 U of the second enzyme. The gel blocks were embedded in a second, slightly thicker gel, and electrophoresis was carried

out. For partial digestion, the DNA agarose pieces were incubated overnight at 4 °C in reaction buffer without magnesium and 10 U of enzyme. After the addition of 10–40 µl 1 mM magnesium chloride, the mixture was incubated for 1 h at the appropriate reaction temperature. The reaction was stopped by adding 5 µl 0.5 M EDTA. PFGE was performed in a contour-clamped homogeneous-electric-field apparatus (CHEF-DR II) from Bio-Rad. Agarose gels were prepared at 1–1.2% in 1 × TAE buffer. Fragments were separated at 14 °C and at a constant voltage of 170 V with pulse times varying with the size range. To check for the existence of fragments smaller than 15 kb, all digests were systematically resolved by conventional gel electrophoresis. *Saccharomyces cerevisiae* chromosomes, bacteriophage lambda DNA concatemers from cI857S7, MidRange PFG Marker I [bacteriophage lambda DNA concatemers from cI857S7 and *Xho*I-digested lambda DNA (New England Biolabs)], and MidRange PFG Marker II [bacteriophage lambda DNA concatemers and *Xba*I-digested lambda DNA (New England Biolabs)] were used as molecular size standards. Gels were stained for 1 h in ethidium bromide (0.5 mg ml⁻¹), destained in water, and photographed under ultraviolet light.

DNA hybridization and genomic DNA probes. Restriction fragments were transferred onto positively charged nylon membranes (Boehringer Mannheim) by a modified Southern transfer procedure (Dubitsky *et al.*, 1992). After electrophoresis, DNA fragments were depurinated in 0.25 M HCl for 10 min and were then denatured in 0.5 M NaOH/1.5 M NaCl for 30 min. A dry membrane was placed on the alkaline gel. For transfer, 20 × SSC (3 M NaCl/0.3 M sodium citrate, pH 7.0) was used. The DNA was bound to the membrane by baking at 120 °C for 30 min. Probes were prepared by random-primed DNA labelling with DIG-High Prime (Boehringer Mannheim). CSPD (disodium 3-(4-methoxyspiro[1,2-dioxetane-3,2'-(5'-chloro)tricyclo[3.3.1.1^{3,7}]decan]-4-yl)phenyl phosphate from Boehringer Ingelheim Bioproducts) was used for chemiluminescent detection of the DNA. Stripping and reprobing were performed as recommended by the membrane manufacturer.

RESULTS

Selection of restriction endonucleases and determination of the genome size

Restriction endonucleases were tested for their ability to digest the *S. carnosus* chromosome into a small number of fragments that could be resolved well by PFGE. Due to the low G + C content (35–36 mol%) of *S. carnosus* (Schleifer & Fischer, 1982), enzymes were tested that recognize G + C-rich sequences. The enzymes *Apa*I, *Not*I, *Sfi*I, *Sse*8387I and *Sma*I each cleaved the genome into a convenient number of fragments with suitable sizes for PFGE. *Not*I, *Sfi*I and *Apa*I produced 3, 3 and 12 restriction fragments, respectively (Table 1, Fig. 1) and were chosen for mapping the genome of *S. carnosus*. The sizes of the largest *Not*I fragment (No1), the largest *Sfi*I fragment (Sf1) and the NoSf1 fragment were determined under different parameters as used for the PFGE gel in Fig. 1 (data not shown). The two smallest fragments of the *Apa*I digest were not observed in PFGE gels, but could be detected by conventional electrophoresis (data not shown). *Sse*8387I and *Sma*I cut the DNA into 13 and 23 fragments, respectively. Further

Fig. 1. PFGE separation of DNA from *S. carnosus* digested with *ApaI*, *NotI*, *SfiI* and *Sse8387I*. Migration parameters: 1% agarose, pulse times from 5 to 65 s at 170 V for 36 h. Lanes: 1, lambda DNA concatemers; 2, *ApaI* digestion; 3, *ApaI*-*NotI* digestion; 4, *ApaI*-*SfiI* digestion; 5, *ApaI*-*NotI*-*SfiI* digestion; 6, MidRange PFG Marker II; 7, *Sse8387I* digestion; 8, *Sse8387I*-*NotI* digestion; 9, *Sse8387I*-*SfiI* digestion; 10, *Sse8387I*-*NotI*-*SfiI* digestion; 11, lambda DNA concatemers; 12, *NotI* digestion; 13, *SfiI* digestion; 14, *NotI*-*SfiI* digestion.

analysis by *Sse8387I* digests was required to arrange some *ApaI* fragments and to confirm the results obtained with *ApaI*, *NotI* and *SfiI*. Among the other restriction enzymes tested, *AscI*, *FseI* and *SgfI* did not cut the genome. *BssHII*, *EclXI* and *KspI* cut the DNA too frequently to be of use for macrophysical mapping.

The size of the *S. carnosus* genome was estimated to be 2590 kb by adding together the sizes of the fragments from each of the *NotI*, *SfiI*, *ApaI* and *Sse8387I* digests (Table 1).

Alignment of *NotI* and *SfiI* fragments in the *S. carnosus* genome

NotI-*SfiI* double digests were performed to locate the restriction sites for *NotI* in relation to those for *SfiI* on the chromosome. After single digestions, *NotI* and *SfiI* each generated three fragments. After double digestion, six fragments were detected (Table 1). These numbers of fragments were consistent with a circular map of the chromosome. Only five electrophoretically distinct bands were produced. The band at about 200 kb

contained two comigrating DNA fragments (Fig. 1). The two largest *NotI* and the two largest *SfiI* fragments disappeared after double digestion, indicating that they contained *SfiI* and *NotI* sites, respectively. By comparing the fragment sizes obtained, one *NotI* site was localized on the *SfiI* fragment, one *SfiI* site on the *NotI* fragment, two *NotI* sites on the *SfiI* fragment, and two *SfiI* sites on the *NotI* fragment. To confirm these results, consecutive double digestions with *NotI* and *SfiI* were analysed by two-dimensional PFGE. The restriction fragments generated, summarized in Table 2, were in accordance with the results obtained by comparing the fragment sizes of the single and double digestions with *NotI* and *SfiI*. Hybridization experiments with various gene probes against DNA digested with *NotI*, *SfiI* and *NotI*-*SfiI* were also performed to identify the overlapping fragments. The hybridization results are given in Table 3. By using the hybridization data and by combinatorial analyses of the fragments obtained after single, double, and consecutive double digestions, we were able to map all of the *NotI* and *SfiI* sites.

Alignment of *ApaI* fragments in the *S. carnosus* genome

ApaI cleaved the *S. carnosus* chromosome into 12 restriction fragments (Table 1). The *Ap11* and *Ap12* fragments could only be detected by conventional electrophoresis and the *Ap8* and *Ap9* fragments formed a doublet. Therefore, only nine distinct bands could be identified (Fig. 1). Double digestions with *ApaI* and *NotI* or *SfiI* and triple digestions with these enzymes were performed to locate the *ApaI* sites in relation to the other restriction sites. In both *ApaI*-*NotI* and *ApaI*-*SfiI* double digestions, 15 fragments were generated; 18 fragments were generated in *ApaI*-*NotI*-*SfiI* triple digestions (Table 1). The *Ap1*, *Ap3* and *Ap8* fragments disappeared in the *NotI*-*ApaI* double digestion (Fig. 1), indicating that each carried one *NotI* site. Similarly the *Ap1* fragment was found to contain one *SfiI* site, and the *Ap2* fragment two *SfiI* sites. Consecutive double digestions of some isolated *ApaI* fragments with *NotI* and *SfiI* (Table 2) and hybridization experiments with various gene probes (Table 3) were also performed. It was possible to map the *Ap1*, *Ap2* and *Ap3* fragments in relation to the *NotI* and *SfiI* restriction sites by combinatorial analysis of the fragments. The *Ap2* and *Ap3* fragments are located next to each other, and the *Ap1* fragment is separated by approximately 60 kb from the *Ap2* fragment. The 52 kb *Ap8* fragment is localized in this gap because it disappeared in the *NotI*-*ApaI* digest (Fig. 1) and two additional fragments with sizes of about 43 kb and 6.5 kb were found.

To determine which of the two smallest *ApaI* fragments, the 7.8 kb *Ap11* or the 5.2 kb *Ap12* fragment, is positioned in the remaining gap of about 8 kb, cross-Southern hybridization was performed. The *Ap11* and *Ap12* fragments were used to probe blots of genomic digests created by *NotI*, *SfiI* and *Sse8387I*. The *Ap12* probe hybridized to a 200 kb *NotI*-*SfiI* fragment (data

Table 1. Restriction fragments of *S. carnosus*

The total sizes of *NotI*, *SfiI*, *NotI-SfiI*, *ApaI*, *ApaI-NotI*, *ApaI-SfiI*, *ApaI-NotI-SfiI* and *Sse8387I* were 2560 ± 112 , 2588 ± 105 , 2583 ± 115 , 2606 ± 78 , 2591 ± 79 , 2614 ± 79 , 2603 ± 83 and 2575 ± 85 kb, respectively.

Name* and size (kb)† of fragments:															
<i>NotI</i>		<i>SfiI</i>		<i>NotI-SfiI</i>		<i>ApaI</i>		<i>ApaI-NotI</i>		<i>ApaI-SfiI</i>		<i>ApaI-NotI-SfiI</i>		<i>Sse8387I</i>	
Fragment	Size	Fragment	Size	Fragment	Size	Fragment	Size	Fragment	Size	Fragment	Size	Fragment	Size	Fragment	Size
No1	1750 ± 90	Sf1	1650 ± 80	NoSf1	1450 ± 80	Ap1	600 ± 16	ApNo1	470 ± 12	ApSf1	510 ± 13	ApNoSf1	415 ± 12	Ss1	540 ± 12
No2	635 ± 16	Sf2	710 ± 18	NoSf2	330 ± 10	Ap2	470 ± 12	ApNo2	435 ± 12	ApSf2	445 ± 12 (Ap3)	ApNoSf2	345 ± 10	Ss2	470 ± 12
No3	175 ± 6	Sf3	228 ± 7	NoSf3	228 ± 7 (Sf3)	Ap3	445 ± 12	ApNo3	415 ± 12 (Ap4)	ApSf3	415 ± 12 (Ap4)	ApNoSf3	335 ± 10	Ss3	395 ± 10
				NoSf4	200 ± 6	Ap4	415 ± 12	ApNo4	330 ± 10	ApSf4	260 ± 8 (Ap5)	ApNoSf4	260 ± 8	Ss4	287 ± 8
				NoSf5	200 ± 6	Ap5	260 ± 8	ApNo5	257 ± 8 (Ap5)	ApSf5	226 ± 7 (Sf3)	ApNoSf5	226 ± 7	Ss5	238 ± 8
				NoSf6	175 ± 6 (No3)	Ap6	132 ± 5	ApNo6	165 ± 6	ApSf6	145 ± 5	ApNoSf6	165 ± 6	Ss6	142 ± 6
						Ap7	124 ± 5	ApNo7	132 ± 5 (Ap6)	ApSf7	132 ± 5 (Ap6)	ApNoSf7	145 ± 5	Ss7	115 ± 6
						Ap8	52 ± 2	ApNo8	124 ± 5 (Ap7)	ApSf8	124 ± 5 (Ap7)	ApNoSf8	130 ± 5	Ss8	97 ± 5
						Ap9	52 ± 2	ApNo9	100 ± 3	ApSf9	100 ± 3	ApNoSf9	125 ± 5	Ss9	83 ± 5
						Ap10	43 ± 2	ApNo10	52 ± 2 (Ap9)	ApSf10	97 ± 3	ApNoSf10	100 ± 3	Ss10	83 ± 5
						Ap11	7·8	ApNo11	43 ± 2 (Ap10)	ApSf11	52 ± 2 (Ap8)	ApNoSf11	100 ± 3	Ss11	62 ± 4
						Ap12	5·2	ApNo12	43 ± 2	ApSf12	52 ± 2 (Ap9)	ApNoSf12	97 ± 3	Ss12	43 ± 3
								ApNo13	7·8 (Ap11)	ApSf13	43 ± 2 (Ap10)	ApNoSf13	52 ± 2	Ss13	20 ± 1
								ApNo14	6·5	ApSf14	7·8 (Ap11)	ApNoSf14	43 ± 2		
								ApNo15	5·2 (Ap12)	ApSf15	5·2 (Ap12)	ApNoSf15	43 ± 2		
												ApNoSf16	7·8		
												ApNoSf17	6·5		
												ApNoSf18	5·2		

* Each restriction fragment was named by the initial letters of the enzyme used to produce it (No, *NotI*; Sf, *SfiI*; Ap, *ApaI*; Ss, *Sse8387I*). Fragments obtained from multiple digestions were also named with the initial letters of the enzymes used (NoSf, *NotI-SfiI*; ApNo, *ApaI-NotI*; ApSf, *ApaI-SfiI*; ApNoSf, *ApaI-NotI-SfiI*). The fragments from each digest were numbered in order, from the largest to the smallest.

† The size indicated is the mean of at least four independent values ± the standard deviation from the mean.

Table 2. Products of consecutive double digestions

First/second enzyme	Restriction fragment	Fragments obtained after the second digestion
<i>NotI/SfiI</i>	No2 (635 ± 16 kb)	225 kb (Sf3), 200 kb (NoSf4), 200 kb (NoSf5)
	No3 (175 ± 6 kb)	175 kb (No3)
<i>SfiI/NotI</i>	Sf1 (1650 ± 80 kb)	1500 kb (NoSf1), 200 kb (NoSf4)
	Sf2 (710 ± 18 kb)	330 kb (NoSf2), 200 kb (NoSf5), 170 kb (No3)
	Sf3 (228 ± 7 kb)	230 kb (Sf3)
<i>ApaI/NotI</i>	Ap1 (600 ± 16 kb)	430 kb (ApNo2), 160 kb (ApNo6)
	Ap3 (445 ± 12 kb)	330 kb (ApNo4), 100 kb (ApNo9)
<i>ApaI/SfiI</i>	Ap1 (600 ± 16 kb)	510 kb (ApSf1), 97 kb (ApSf9)
	Ap2 (470 ± 12 kb)	220 kb (Sf3), 145 kb (ApSf5), 100 kb (ApSf8)
<i>Sse8387I/ApaI</i>	Ss1 (540 ± 12 kb)	270 kb, 260 kb
	Ss2 (470 ± 12 kb)	260 kb, 120 kb, 70 kb
	Ss3 (395 ± 10 kb)	360 kb, 30 kb

Table 3. Assignment of genetic markers to the physical map of *S. carnosus*

Probe	Gene function	Hybridizing fragments	Reference*
<i>ald</i> genes	Aldehyde dehydrogenases	No1, Sf1, NoSf1, Ap1, ApNo3, ApSf10, ApNoSf11, Ss1, SsSf3	K. Nikoleit, unpublished
<i>fda</i>	Fructose-1,6-bisphosphate aldolase	No1, Sf1, NoSf1, Ap1, ApNo3, ApSf10, ApNoSf11, Ss1, SsSf3	Witke & Götz (1993)
<i>glcA, glcB</i>	EII ^{Glc} 1, EII ^{Glc} 2	No1, Sf1, NoSf1, Ap3, ApNo4, ApNoSf3, Ss3	Christiansen & Hengstenberg (1996)
<i>lsp</i>	Signal peptidase II	No1, Sf1, NoSf1, Ap3, ApNo4, ApNoSf3, Ss2	Witke & Götz (1995)
<i>moe</i>	Molybdenum cofactor biosynthesis gene cluster	No1, Sf1, NoSf1, Ap4, Ss1, SsSf3	H. Neubauer and others, unpublished
<i>mtlD, mtlF, mtlA</i>	Mannitol-1-phosphate dehydrogenase, EIII ^{Mtl} , EII ^{Mtl}	No1, Sf1, NoSf1, Ap10, Ss6	Fischer & Hengstenberg (1992)
<i>nir/nar</i>	Nitrite reduction gene cluster/nitrate reductase	No1, Sf1, NoSf1, Ap4, Ss1, SsSf3	I. Pantel and others, unpublished
<i>ptsH, ptsI</i>	HPr, enzyme I	No2, Sf1, NoSf4, Ap3, ApNo9, Ss3	Kohlbrecher <i>et al.</i> (1992)
<i>rplM, rpsI</i>	Ribosomal proteins L13, S9	No1, Sf1, NoSf1, Ap4, Ss8	Overhoff-Freundlieb & Freudel (1991)
rRNA genes	16S rRNA, 23S rRNA	No3, Ap4, Ap6, Ap8, Ss5, Ss6	P. Nerf & F. Götz, unpublished
<i>sceA</i>	Exoprotein	No1, Sf1, NoSf1, Ap9, Ss6	B. Krismer and others, unpublished
<i>sceB</i>	Exoprotein	No1, Sf1, NoSf1, Ap4, Ss8	B. Krismer and others, unpublished
<i>secA</i>	Protein export	No2, Sf3, Ap2, Ss4	Klein <i>et al.</i> (1995)
<i>secE, nusG, rplK</i>	Protein export, termination protein, ribosomal protein L11	No2, Sf2, NoSf5, Ap2, ApSf6, Ss13	Meens <i>et al.</i> (1994)
<i>secY</i>	Protein export	No1, Sf1, NoSf1, Ap4, Ss8	Tschauder <i>et al.</i> (1992)

* All the unpublished work cited here was done at the Lehrstuhl für Mikrobielle Genetik, Universität Tübingen.

not shown) and to the 238 kb Ss5 fragment (Fig. 2). No hybridization signal was obtained with *NotI*- or *SfiI*-digested DNA. Because of the occurrence of two 200 kb *NotI*-*SfiI* fragments we could not localize Ap12. Therefore the arrangement of some *Sse8387I* fragments was determined by analysing double digestions with *NotI* and *SfiI*. *Sse8387I* cleaved the *S. carnosus* chromosome into 13 restriction fragments (Table 1). Only 12 distinct bands appeared on the gel because the Ss9 and Ss10 fragments formed a doublet (Fig. 1). We could show that the Ss5 fragment carried two *NotI* sites and is therefore localized in the region of No3. The Ss3 fragment contained one *NotI* site and therefore overlaps with the No1 and No2 fragments. The Ss1 fragment carried one *SfiI* site and represented the overlapping fragment of Sf1 and Sf2. Two *SfiI* sites were found on the Ss4 fragment. Information on the hybridization of probes was important in verifying the arrangement of these fragments. Consecutive double digestions of specific *Sse8387I* fragments with *ApaI* were also performed. The sizes of the resulting fragments are given in Table 2. The digestion of the Ss1 fragment with *ApaI* yielded two fragments, of about 270 and 260 kb. The 270 kb *ApaI*-*Sse8387I* fragment and the Ss1 fragment showed (part of) the same hybridization pattern as the Ap4

fragment. This indicated that the Ss1 fragment is positioned in the region of the Ap4 fragment and next to the Ap1 fragment, which also showed (part of) the same hybridization pattern as Ss1. The Ss2 fragment was aligned next to the Ss3 fragment, which is located in the region of Ap3, because the *lsp* gene probe hybridized to both the Ss2 and the Ap3 fragment, showing that these were overlapping fragments.

The arrangement of *ApaI* fragments in the region of the Ss2 fragment was also determined by consecutive double digestions with *ApaI*. Fragments were obtained with sizes of about 260, 120 and 70 kb (Table 2). The 70 kb fragment represented the overlapping *ApaI*-*Sse8387I* fragment, which hybridized to the *lsp* gene probe. The order of the 260 kb and the 124 kb fragments, representing Ap5 and Ap7, was determined by partial digestion of the DNA with *ApaI* in combination with hybridization experiments. The *lsp* gene hybridized to the Ap3 fragment (445 kb) obtained by complete digestion with *ApaI*. With DNA partially digested with *ApaI* the *lsp* gene hybridized to a 570 kb fragment (data not shown), a finding which showed that the Ap7 fragment (124 kb) is positioned next to Ap3 and Ap5 next to Ap7 on the chromosome.

Fig. 2. DNA from *S. carnosus* digested with *Sse8387I*, separated by PFGE, blotted onto nylon membranes, and hybridized with different probes. Migration parameters: 1% agarose, pulse times from 5 to 65 s at 170 V for 38 h. Lanes: 1, MidRange PFG Marker I; 2, *Sse8387I*-digested DNA; 3, hybridization of lane 2 with the 5.2 kb Ap12 fragment used as a probe; 4, hybridization of lane 2 with the 7.8 kb Ap11 fragment used as a probe.

The location of Ap6 (132 kb) and Ap10 (43 kb) in the gap between Ap5 (260 kb) and Ap4 (415 kb) was also determined by *ApaI* partial digestion and hybridization with the *sceA* gene probe. The *sceA* gene is localized on the Ap9 fragment (52 kb) obtained by complete digestion with *ApaI*. After partial digestion, the *sceA* probe hybridized to 185 kb and 450 kb fragments (Fig. 3a), representing the Ap9-Ap6 and Ap9-Ap6-Ap5 partial digestion products, respectively. This showed the neighbouring position of the Ap9 and Ap6 fragment and confirmed the order of Ap5 (260 kb) and Ap7 (124 kb) also determined by partial digestion with *ApaI*. The *mtlD-mtlF-mtlA* genes hybridized to the 43 kb Ap10 fragment obtained after complete digestion with *ApaI*. After partial digestion with *ApaI*, these probes hybridized to a 460 kb fragment (data not shown). We also obtained a hybridization signal at 460 kb with the *sceB* probe (Fig. 3a), which is localized on the Ap4 fragment (415 kb). The 460 kb partial digestion product contained the Ap4 and Ap10 fragments. These results demonstrated that the 43 kb Ap10 fragment and the Ap4 fragment are positioned next to each other on the chromosome. The hybridization signal at > 800 kb (Fig. 3a) represented the partial digestion product Ap10-Ap4-Ap1.

The alignment of the remaining Ap11 fragment was performed by cross-Southern hybridization. A hybridization signal appeared only with *Sse8387I*-digested DNA, at about 142 kb (Fig. 2). This fragment (Ss6) was also mapped by partial digestion and hybridization with various gene probes. The *sceA* probe hybridized to the 142 kb Ss6 fragment generated by complete digestion with *Sse8387I*, and to 240 kb and 360 kb fragments from partial digestions with *Sse8387I* (Fig. 3b). This indicated that Ss6 is positioned next to Ss8 (97 kb) and Ss7 (115 kb). The identical arrangement of these fragments was obtained in hybridization experiments with other gene probes. The alignment of these fragments and the cross-hybridization of the 7.8 kb Ap11 fragment to Ss6 revealed the localization of Ap11 in the remaining small gap between Ap10 (43 kb) and Ap9 (52 kb). The resulting physical map with *NotI*, *SfiI*, and *ApaI* sites is presented in Fig. 4. The *Sse8387I* fragments Ss1, Ss2, Ss3, Ss5, Ss6, Ss7 and Ss8 are also shown.

Location of genetic markers on the chromosome

Fifteen previously identified genes and gene clusters from *S. carnosus* were localized by Southern hybridization on the physical map (Fig. 4). The markers used for genetic mapping are described in Table 3. Fragments of the encoding regions of each of these genes or gene clusters were hybridized to digests of *S. carnosus* DNA, thereby defining the loci on the chromosome. All genetic markers were associated with specific restriction fragments. Hybridizing fragments are summarized in Table 3. The hybridization with some of the gene probes was also indispensable for the alignment of the *NotI*, *SfiI* and *ApaI* fragments. Southern hybridization with probes corresponding to the 16SrRNA gene and to the 5' end of the 23S rRNA gene were used to map the restriction fragments that contain the rRNA genes. With these probes we could identify four *rrn* loci clustered in two different regions of the chromosome. They were positioned on the Ap4, No3, Ap8 and Ap6 fragments. The 23S rRNA gene of *S. carnosus* contained an *ApaI* site (Ludwig *et al.*, 1992). Therefore the *rrn* genes could be localized at the junctions of Ap4-Ap10, Ap6-Ap5, Ap1-Ap8 and Ap8-Ap12.

DISCUSSION

By PFGE analysis, the chromosome size of *S. carnosus* was estimated to be 2590 kb. This value is in the same order of magnitude as the size of 2750 kb determined for *S. aureus* (Pattee *et al.*, 1990). The genomes of bacteria vary in size, from 600 kb for *Mycoplasma genitalium* to 9450 kb for *Myxococcus xanthus* (Cole & Saint Girons, 1994). The *S. carnosus* chromosome could be placed among the larger genomes in group 2 with a size of 1500–3000 kb. Micro-organisms with this genome size exist in the Archaeobacteria and in various Eubacteria such as *Campylobacter*, *Enterobacter*, *Haemophilus*, *Helicobacter*, *Lactococcus*, *Neisseria* and *Streptococcus* (Cole & Saint Girons, 1994; Fonstein & Haselkorn, 1995).

Fig. 3. DNA from *S. carnosus* digested with *Apal* or *Sse8387I*, separated by PFGE, blotted onto nylon membranes, and hybridized with various gene probes. (a) Migration parameters: 1% agarose, pulse times from 10 to 100 s at 170 V for 38 h. Lanes: 1, lambda DNA concatemers; 2, partial *Apal* digestion; 3, complete *Apal* digestion; 4, hybridization of DNA fragments in lane 2 with the *sceA* probe; 5, hybridization of DNA fragments in lane 3 with the *sceA* probe; 6, hybridization of DNA fragments in lane 2 with the *sceB* probe; 7, hybridization of DNA fragments in lane 3 with the *sceB* gene probe. (b) Migration parameters: 1% agarose, pulse times from 10 to 100 s at 170 V for 36 h. Lanes: 1, lambda DNA concatemers; 2, partial *Sse8387I* digestion; 3, complete *Sse8387I* digestion; 4, hybridization of DNA fragments in lane 2 with the *sceA* gene probe; 5, hybridization of DNA fragments in lane 3 with the *sceA* probe.

Fig. 4. Physical and genetic map of *S. carnosus* TM300 generated with *Apal*, *NotI*, *SfiI* and *Sse8387I*. The outer circle represents *Apal* fragments, the second circle *NotI* fragments, and the third circle *SfiI* fragments. The *Sse8387I* fragments Ss1, Ss2, Ss3, Ss5, Ss6, Ss7 and Ss8 represent part of the inner circle. Restriction fragments are numbered in order of their sizes (Table 1). Segments of the inner circle which are not numbered are not yet mapped. The locations of the genes are indicated by the arc lines outside the circle. For gene designations, see Table 3

The physical map of *S. carnosus* was established by a combination of complementary techniques. The analyses of single, multiple and partial digests in combination

with hybridization experiments with genetic markers and cross-Southern hybridization allowed the alignment of the *NotI*, *SfiI* and *ApaI* fragments. Consecutive double digestions were used to confirm the localization of the fragments. This technique was also necessary for the unequivocal mapping of the *ApaI* fragments to locate some of the *Sse8387I* restriction sites. The data obtained were consistent with the concept of a single and circular chromosome in *S. carnosus*.

Both cross-Southern hybridization and consecutive double digestions analysed by two-dimensional electrophoretic separation of the fragments were affected by technical difficulties. Cross-Southern hybridization, in which labelled fragments produced by one enzyme are hybridized to fragments produced by another, often gave ambiguous results. The fragments to be labelled could not be obtained in sufficient purity because there was often a contaminating smear of degraded DNA in gel electrophoretograms. We therefore had to repeat these experiments several times with different DNA preparations to obtain unequivocal results. No1, Sf1 and NoSf1 were underrepresented in electrophoretograms because some slight non-specific DNA degradation probably occurred during extraction and incubation with the restriction enzyme. Another explanation may be that the DNA was isolated from exponentially growing bacteria containing replication forks. The relative amounts of the smaller fragments should be less susceptible to these interfering factors than those of the larger DNA fragments, since they offer a smaller target. The underrepresentation of the larger fragments may also be a reason for the failure to localize the Ap11 fragment on No1, Sf1 and NoSf1, and Ap12 on No2 and Sf2 by cross-Southern hybridization.

Eighteen genetic loci were placed on the *S. carnosus*

genome map. Four *rrn* loci were found to be clustered in two regions separated by approximately 800 kb. *rrn* locus grouping has been observed in many bacteria, such as *Escherichia coli* (Bachmann, 1990), *Bacillus subtilis* (Piggot, 1990), *Lactococcus lactis* subsp. *lactis* (Tulloch *et al.*, 1991), *Clostridium perfringens* (Canard & Cole, 1989), *Haemophilus influenzae* (Lee *et al.*, 1989) and *Streptococcus thermophilus* A054 (Roussel *et al.*, 1994). The *rrn* loci of *S. carnosus* were located in a region representing about half of the chromosome.

Many genes were found on the 415 kb Ap4 fragment. In a first approach to map this region in greater detail, we determined the location of the *Sse8387I* sites. The *nir/nar* genes, involved in nitrite and nitrate reduction, and the *moe* genes, involved in the biosynthesis of the molybdenum cofactor, were located on a 290 kb *ApaI*–*Sse8387I* fragment. The *sceB* and *secY* genes were situated on the 97 kb *Ss8* fragment. The genes *rplM*–*rpsI*, encoding ribosomal proteins, are organized in an operon, which was also mapped on *Ss8*. The *rplK* gene, encoding the ribosomal protein L11, was positioned together with *secE* and *nusG* on a 150 kb *SfiI*–*ApaI* fragment. The *secA* gene was found on the neighbouring 220 kb *SfiI* fragment (*Sf3*).

The construction of a genomic map of the *S. carnosus* chromosome is a significant advance in the genetic analysis of this industrially important organism. Newly identified genes can now be easily mapped on the chromosome. By the use of additional restriction endonucleases and subcloning, regions of particular interest could be mapped in greater detail.

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