RT-PCR as a tool for systematic transcriptional analysis of large regions of the *Bacillus subtilis* genome

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Transcriptional analysis of five different regions of the *Bacillus subtilis* 168 genome, comprising a total of 175 kb encoding newly identified genes, was carried out using the RT-PCR technique as part of the functional analysis of the whole genome of this bacterium. Amplification of mRNA fragments allowed the detection of both highly and poorly transcribed genes covering 81% of putative ORFs, and also the monitoring of variations in the expression level among genes differentially expressed during particular bacterial growth phases.

Keywords: *Bacillus subtilis*, transcription, new genes

INTRODUCTION

Studies of the expression of large genomic regions in bacteria are technically difficult since until recently simultaneous analysis of coordinated expression was only possible for a limited subset of genes. Most of the currently available methodology for studying differential expression of genes is applicable only to eukaryotic poly(A)-tailed mRNA; the absence of polyadenylation in prokaryotic mRNA does not help in bacterial transcriptional analysis. Only 4–5% of the total bacterial RNA preparation is mRNA, which adds a further element of considerable difficulty in detecting transcription of bacterial genes, particularly of those that are poorly expressed or that have highly unstable messengers.

A rRNA subtraction approach has been used to identify differentially expressed bacterial genes (Plum & Clark-Curtiss, 1994) but, after removal of rRNA, it is difficult to get clean mRNA preparations without selective loss of mRNA. Methods relying on differential hybridization to overlap moderately large clones of the bacterial genome (Chuang *et al.*, 1993) require subcloning followed by sequencing of the relevant genes. Monitoring expression of a large number of genes in eukaryotes has been possible by using DNA arrays or oligonucleotide microarrays (Pease *et al.*, 1994; Lockhart *et al.*, 1996; Wodicka *et al.*, 1997). Hybridization of labelled cDNA or mRNA to the high-density probe microarrays allowed the analysis of expression of many genes in a single hybridization experiment. Due to the high back-

ground that a probe of labelled total RNA would give, DNA or oligonucleotide microarray technology may not be applicable for bacterial transcriptional analysis. Nevertheless, the method has been adapted to monitor gene expression in *Streptococcus pneumoniae* (de Sazieu *et al.*, 1997) with promising results, but it requires hybridization to probes labelled either by radioactivity or otherwise. Here, we demonstrate the applicability of specific amplification of mRNA fragments; the mRNA complementary strand is synthesized in the presence of reverse transcriptase followed by several rounds of amplification by PCR using appropriate sets of oligonucleotides. The method needs as little as 50 ng total RNA, does not require the use of labelled probes in hybridization experiments and allows monitoring of the presence of different levels of RNA.

METHODS

Standard media and DNA manipulations. *Bacillus subtilis* 168 (*trpC2*) was the strain used in the experiments and was provided by C. Anagnostopoulos (INRA, Jouy-en-Josas, France). The procedures for the growth of *B. subtilis* and DNA manipulation were as described by Harwood & Cutting (1990). DNA was isolated from *B. subtilis* cultures grown at 37 °C in minimal medium, nutrient broth and sporulation medium as described by Harwood & Cutting (1990) and Schaeffer *et al.* (1965). MM contains Spizizen minimal salts (Anagnostopoulos & Spizizen, 1961) supplemented with 0.4% (w/v) glucose, 0.2% (w/v) L-glutamine, 50 g L-tryptophan l⁻¹ and trace elements (Harwood & Cutting, 1990) containing 4 mg FeCl₃ l⁻¹ and 0.2 µg MnSO₄ l⁻¹ instead of FeCl₂ and MnCl₂, respectively, adjusted to pH 7.0. NB contains 8 g

Table 1. Primers used for RT-PCR

f, Forward primer; r, reverse primer.

936.6–976.6 kb				1265.4–1280 kb		2744–2767 kb	
Gene/ primer	Coordinates	Gene/ primer	Coordinates	Gene/ primer	Coordinates	Gene/ primer	Coordinates
<i>yfbR-f</i>	936859–936883	<i>ygbA-f^h</i>	962033–962057	<i>yjcP-f</i>	1266242–1266261	<i>aapA-f</i>	2766389–2766413
<i>yfbR-r</i>	937136–937112	<i>ygbA-r</i>	962321–962297	<i>yjcP-r</i>	1266478–1266457	<i>aapA-r</i>	2766762–2766786
<i>ygaB-f</i>	937749–937773	<i>ygaM-f</i>	962915–962939	<i>yjcQ-f</i>	1266645–1266666	<i>yraA-f</i>	2756759–2756783
<i>ygaB-r</i>	938090–938066	<i>ygaM-r</i>	963208–963184	<i>yjcQ-r</i>	1266854–1266832	<i>yraA-r</i>	2757223–2757199
<i>ygaC-f</i>	938237–938261	<i>ygcA-f</i>	962915–962939	<i>yjcR-f</i>	1267147–1267168	<i>adhA-f</i>	2755599–2755622
<i>ygaC-r</i>	938551–938527	<i>ygcA-r</i>	964663–964639	<i>yjcR-r</i>	1267461–1267430	<i>adhA-r</i>	2756062–2756049
<i>ygaD-f</i>	939486–939510	<i>ygaN-f</i>	964938–964962	<i>yjdA-f</i>	1268505–1268526	<i>yraB-f</i>	2754991–2754968
<i>ygaD-r</i>	939986–939962	<i>ygaN-r</i>	965199–965175	<i>yjdA-r</i>	1268896–1268873	<i>yraB-r</i>	2754724–2754703
<i>ygaE-f</i>	941361–941337	<i>yhzA-f^h</i>	965418–965442	<i>yjdB-f</i>	1269464–1269446	<i>yraC-r</i>	2754674–2754650
<i>ygaE-r</i>	941020–941044	<i>yhzA-r</i>	965684–965660	<i>yjdB-r</i>	1269295–1269314	<i>yraC-f^h</i>	2754410–2754434
<i>ygaF-f</i>	943396–943420	<i>ygaO-f^h</i>	966178–966154	<i>yjdC-f^h</i>	1270862–1270883	<i>yraD-r</i>	2754332–2754308
<i>ygaF-r</i>	943635–943611	<i>ygaO-r</i>	965708–965732	<i>yjdC-r</i>	1271210–1271189	<i>yraD-f</i>	2754169–2754193
<i>ygaG-f</i>	944091–944115	<i>yhbA-f</i>	967753–967777	<i>yjdD-f</i>	1272698–1272725	<i>adhB-f</i>	2752832–2752855
<i>ygaG-r</i>	944338–944314	<i>yhbA-r</i>	968103–968079	<i>yjdD-r</i>	1272994–1272984	<i>adhB-r</i>	2753277–2753254
<i>ygaH-f</i>	944671–944695	<i>yhbB-f^h</i>	967361–967337	<i>yjdE-f</i>	1274574–1274600	<i>yraE-f</i>	2754039–2754015
<i>ygaH-r</i>	945000–944976	<i>yhbB-r</i>	966741–966765	<i>yjdE-r</i>	1274857–1274837	<i>yraE-r</i>	2753780–2753803
<i>ygaA-f</i>	945302–945326	<i>yhbB-f</i>	969035–969059	<i>yjdF-f</i>	1275390–1275408	<i>yraF-f</i>	2752603–2752581
<i>ygaA-r</i>	945620–945596	<i>yhbB-r</i>	969308–969284	<i>yjdF-r</i>	1275658–1275640	<i>yraF-r</i>	2752430–2752451
<i>ygaI-f</i>	953655–953631	<i>cspr-f^h</i>	969644–969668	<i>yjdG-f</i>	1276216–1276192	<i>yraG-f</i>	2752187–2752163
<i>ygaI-r</i>	952882–952906	<i>cspr-r</i>	970123–970099	<i>yjdG-r</i>	1275929–1275952	<i>yraG-r</i>	2752030–2752054
<i>ygzA-f^h</i>	953797–953821	<i>yhbD-f^h</i>	970349–970373	<i>yjdH-f</i>	1276860–1276836	<i>yraH-f</i>	2751717–2751693
<i>ygzA-r</i>	953997–953973	<i>yhbD-r</i>	970654–970630	<i>yjdH-r</i>	1276634–1276656	<i>yraH-r</i>	2751548–2751566
<i>ygaJ-f</i>	954640–954642	<i>yhbE-f</i>	971084–971108	<i>yjdI-f</i>	1277251–1277272	<i>yraI-f</i>	2750949–2750925
<i>ygaJ-r</i>	954920–954896	<i>yhbE-r</i>	971379–971355	<i>yjdI-r</i>	1277534–1277513	<i>yraI-r</i>	2750630–2750616
<i>thiA-f</i>	955403–955427	<i>yhbF-f^h</i>	971760–971784	<i>yjdI-r</i>	1278267–1278240	<i>yraJ-f</i>	2750421–2750399
<i>thiA-r</i>	957172–957148	<i>yhbF-r</i>	972170–972146	<i>yjdJ-f</i>	1278144–1278164	<i>yraJ-r</i>	2750260–2750240
<i>ygaK-f</i>	957601–957625	<i>yhbH-f</i>	975004–975028	<i>yjdK-f^h</i>	1279755–1279729	<i>yraK-f</i>	2748987–2748965
<i>ygaK-r</i>	958060–958036	<i>yhbH-r</i>	975313–975289	<i>yjdK-r</i>	1279259–1279284	<i>yraK-r</i>	2749282–2749260
<i>ygaL-f^h</i>	961165–961189					<i>yraL-f</i>	2748965–2748987
<i>ygaL-r</i>	961470–961446					<i>yraL-r</i>	2749260–2749282
						<i>csn-f</i>	2747875–2747852
						<i>csn-r</i>	2747596–2747618
						<i>yraM-f</i>	2746819–2746796
						<i>yraM-r</i>	2746422–2746401
						<i>yraN-f</i>	2745259–2745282
						<i>yraN-r</i>	2745629–2745651
						<i>yraO-f</i>	2744476–2744498
						<i>yraO-r</i>	2744092–2744114

Difco Bacto Nutrient Broth l⁻¹ supplemented with 0.25 g MgSO₄·7H₂O l⁻¹, 1 g KCl l⁻¹, 0.1 mM MnCl₂, 1 µM FeSO₄ and 1 mM CaCl₂, adjusted to pH 7.1. For induction of the *ara* operon, *B. subtilis* cells were grown in minimal C medium (Pascal *et al.*, 1971) supplemented with 1% (w/v) casein hydrolysate, and L-arabinose or glucose was added to the cultures as carbon sources at a final concentration of 0.4% (w/v).

Transcriptional analysis. Aliquots from the different cultures were lysed (Mellado *et al.*, 1981) and total RNA was extracted at the times indicated in the Results and Discussion section as described by Kedzierski & Porter (1991). Total RNA from sporulating cells was prepared in the same manner but a grinding step with glass beads (2 mm diameter) was used to break the sporulating cells before lysis. When necessary, RNA preparations were rendered DNA-free by incubation with DNase-RNase free (Promega), following the supplier's manual. RT-PCR analysis was carried out by incubating the amounts of total RNA preparations indicated in the Results and Discussion section first with AMV reverse transcriptase, and then with *Thermus flavus* DNA polymerase. The Promega Access RT-PCR system kit containing both enzymes was used, following the supplier's manual. RT-PCR reactions were carried out in a final volume of 100 µl using the oligonucleotides indicated in Table 1 as primers. RT-PCR reactions

were carried out in automated thermocyclers (PTC-100, MJ Research) by incubation at 48 °C for 45 min followed by 2 min heating at 95 °C. Amplifications were carried out by 40 cycles of incubation at 94 °C (0.5 min), 60 °C (1 min) and 68 °C (2 min), with a final extension step of incubation at 68 °C for 7 min. The amplified DNA fragments were analysed by 1.5% (w/v) agarose gel electrophoresis; up to 108 samples were run at the same time in a Scie-Plas electrophoresis unit.

RESULTS AND DISCUSSION

Transcriptional analysis by RT-PCR

Five different regions of the *B. subtilis* genome, 936.6–976.6 kb, 1265.4–1280 kb, 2744–2767 kb, 2854.4–2964.4 kb and 3384–3430 kb (numbering according to Kunst *et al.*, 1997), were chosen as large genomic regions to conduct simultaneous transcriptional analysis of newly identified genes. The five regions encompass a total of 175 kb of new sequence and comprise up to 181 ORFs. To assess if all the putative new genes deduced from the sequencing analysis of the bacterial genome did correspond to functional genes, we needed to develop methods to allow fast, easy and sensitive global trans-

Table 1 (cont.)

2854-4-2964-4 kb						3384-3430 kb			
Gene/ primer	Coordinates	Gene/ primer	Coordinates	Gene/ primer	Coordinates	Gene/ primer	Coordinates	Gene/ primer	Coordinates
<i>dnaB</i> -f	2964133-2964109	<i>ysbB</i> -r	2924374-2924398	<i>ilvN</i> -f	2894342-2894618	<i>yvgO</i> -f	3426885-3426909	<i>yvrG</i> -f [*]	3407253-3407235
<i>dnaB</i> -r	2963768-2963743	<i>ysbB</i> -f	2924594-2924571	<i>ilvN</i> -r	2893893-2893917	<i>yvgO</i> -r	3427175-3427151	<i>yvrG</i> -r	3406826-3406844
<i>ytxB</i> -f	2962020-2961996	<i>yslA</i> -f	2907791-2907815	<i>ilvC</i> -f	2893694-2893670	<i>yvsA</i> -f	3427062-3427044	<i>yvrE</i> -f	3405402-3405390
<i>ytxB</i> -r	2961746-2951722	<i>yslA</i> -r	2908089-2908066	<i>ilvC</i> -r	2892881-2892905	<i>yvsA</i> -r	3426915-3426933	<i>yvrE</i> -r	3405079-3405097
<i>ytxC</i> -f	2960366-2960343	<i>ysbC</i> -f [*]	2923511-2923491	<i>leuA</i> -f	2892390-2892412	<i>yvgN</i> -f [*]	3426546-3426522	<i>yvrD</i> -r	3404596-3404572
<i>ytxC</i> -r	2960054-2960078	<i>ysbC</i> -r	2923009-2923032	<i>leuA</i> -r	2891897-2891919	<i>yvgN</i> -r	3425956-3425981	<i>yvrD</i> -f	3404047-3404071
<i>ysaA</i> -f	2957721-2957739	<i>ysbD</i> -f	2921450-2921428	<i>leuB</i> -f	2890827-2890803	<i>yvgM</i> -r	3425730-3425706	<i>yvrC</i> -f	3403353-3403335
<i>ysaA</i> -r	2958065-2958047	<i>ysbD</i> -r	2920989-2921011	<i>leuB</i> -r	2890396-2890420	<i>yvgM</i> -f	3425400-3425424	<i>yvrC</i> -r	3403052-3403070
<i>lytS</i> -f	2957254-2957236	<i>ysbE</i> -f	2919943-2919919	<i>leuC</i> -f	2889609-2889585	<i>yvgL</i> -r	3424916-3424892	<i>yvrB</i> -f [*]	3402423-3402399
<i>lytS</i> -r	2956957-2956975	<i>ysbE</i> -r	2919752-2919776	<i>leuC</i> -r	2889154-2889177	<i>yvgL</i> -f	3424403-3424427	<i>yvrB</i> -r	3402000-3402024
<i>lytT</i> -f	2955537-2955519	<i>lcfA</i> -f	2918895-2918873	<i>leuD</i> -f	2888537-2888513	<i>yvgK</i> -f	3423931-3423913	<i>yvrA</i> -f [*]	3401092-3401068
<i>lytT</i> -r	2955092-2955110	<i>lcfA</i> -r	2918354-2918376	<i>leuD</i> -r	2888225-2888221	<i>yvgK</i> -r	3423605-3423623	<i>yvrA</i> -r	3400706-3400730
<i>ysbA</i> -f	2954783-2954759	<i>ysiA</i> -f	2917607-2917584	<i>ysaA</i> -f	2887542-2887522	<i>yvgJ</i> -f [*]	3421871-3421889	<i>yvgK</i> -f	3399985-3399967
<i>ysbA</i> -r	2954382-2954406	<i>ysiA</i> -r	2917322-2917345	<i>ysaA</i> -r	2887190-2887212	<i>yvgJ</i> -r	3422113-3422095	<i>yvgK</i> -r	3399808-3399826
<i>ysbB</i> -f	2953397-2953975	<i>ysiB</i> -r	2916441-2916465	<i>ysaC</i> -f	2884247-2884221	<i>yvsG</i> -f [*]	3420935-3420917	<i>yvgJ</i> -f	3399265-3399247
<i>ysbB</i> -r	2953656-2953678	<i>ysiB</i> -f	2916759-2916735	<i>ysaC</i> -r	2883999-2884023	<i>yvsG</i> -r	3421150-3421168	<i>yvgJ</i> -r	3398874-3398892
<i>yscA</i> -f	2952604-2952627	<i>etfB</i> -f	2916145-2916121	<i>lonA</i> -f	2881007-2880983	<i>yvsH</i> -f	3419903-3419885	<i>yvgI</i> -f	3397962-3397944
<i>yscA</i> -r	2952850-2952831	<i>etfB</i> -r	2915728-2915752	<i>lonA</i> -r	2880687-2880711	<i>yvsH</i> -r	3419673-3419691	<i>yvgI</i> -r	3397732-3397750
<i>yscB</i> -r	2953390-2953368	<i>etfA</i> -f	2915143-2915121	<i>ysx</i> -f	2879425-2879401	<i>fluD</i> -r	3418408-3418432	<i>yvgH</i> -f	3397452-3397434
<i>yscB</i> -f	2953081-2953104	<i>etfA</i> -r	2914791-2914812	<i>ysx</i> -r	2879048-2879072	<i>fluD</i> -f	3417832-3417858	<i>yvgH</i> -r	3397120-3397138
<i>rpml</i> -f	2951981-2951957	<i>asd</i> -f	2913628-2913610	<i>ysx</i> -D	2878759-2878737	<i>fluB</i> -f [*]	3417288-3417312	<i>yvgG</i> -f	3396539-3396521
<i>rpml</i> -r	2951726-2951749	<i>asd</i> -r	2913254-2913271	<i>ysx</i> -D	2878483-2878507	<i>fluB</i> -r	3416497-3416521	<i>yvgG</i> -r	3396198-3396216
<i>rplT</i> -f	2951586-2951563	<i>yslB</i> -f	2908202-2908225	<i>hemA</i> -f [*]	2878063-2878039	<i>fluG</i> -f [*]	3416263-3416239	<i>yvgF</i> -f	3395706-3395688
<i>rplT</i> -r	2951388-2951368	<i>yslB</i> -r	2908419-2908396	<i>hemA</i> -r	2877151-2877175	<i>fluG</i> -r	3415335-3415359	<i>yvgF</i> -r	3395368-3395386
<i>ysdA</i> -r	2951057-2951077	<i>sdbC</i> -f	2907730-2907706	<i>hemX</i> -f [*]	2876774-2876751	<i>fluC</i> -f [*]	3415220-3415196	<i>yvgE</i> -f	3395099-3395081
<i>ysdA</i> -f	2951216-2951193	<i>sdbC</i> -r	2907448-2907473	<i>hemX</i> -r	2876113-2876137	<i>fluC</i> -r	3414567-3414591	<i>yvgE</i> -r	3394903-3394921
<i>ysdB</i> -f	2950936-2950912	<i>sdbA</i> -f	2906450-2906426	<i>hemC</i> -f [*]	2875974-2875951	<i>yvrP</i> -f [*]	3414175-3414157	<i>yvgD</i> -f	3394235-3394253
<i>ysdB</i> -r	2950656-2950677	<i>sdbA</i> -r	2906072-2906096	<i>hemC</i> -r	2875129-2875153	<i>yvrP</i> -r	3413817-3413835	<i>yvgD</i> -r	3394537-3394519
<i>ysdC</i> -f	2949951-2949933	<i>sdbB</i> -f	2905250-2905226	<i>hemD</i> -f [*]	2875036-2875012	<i>yvrO</i> -f	3413080-3413062	<i>yvgC</i> -f	3394038-3394020
<i>ysdC</i> -r	2949631-2949649	<i>sdbB</i> -r	2904888-2904912	<i>hemD</i> -r	2874279-2874304	<i>yvrO</i> -r	3412837-3412855	<i>yvgC</i> -r	3393829-3393811
<i>abnA</i> -f	2948812-2948795	<i>ysmA</i> -f	2904528-2904505	<i>hemB</i> -f [*]	2874094-2874069	<i>yvrN</i> -f	3412419-3412401	<i>gerAC</i> -r	3393446-3393422
<i>abnA</i> -r	2948494-2948512	<i>ysmA</i> -r	2904237-2904261	<i>hemB</i> -r	2873587-2873611	<i>yvrN</i> -r	3412223-3412241	<i>gerAC</i> -f [*]	3392441-3392465
<i>araL</i> -f	2944016-2943998	<i>gerE</i> -f	2904034-2904013	<i>hemL</i> -f	2872982-2872957	<i>yvrM</i> -f	3411890-3411872	<i>gerAB</i> -r	3392348-3392324
<i>araL</i> -r	2943532-2943550	<i>gerE</i> -r	2903825-2903849	<i>hemL</i> -r	2872376-2872399	<i>yvrM</i> -r	3411699-3411717	<i>gerAB</i> -f [*]	3391363-3391387
<i>abfA</i> -f	2938101-2938118	<i>ysmB</i> -f	2903469-2903445	<i>spoVID</i> -f	2871220-2871196	<i>yvrL</i> -f [*]	3411089-3411065	<i>gerAA</i> -r	3391047-3391024
<i>abfA</i> -r	2938425-2938407	<i>ysmB</i> -r	2903211-2903235	<i>spoVID</i> -r	2870898-2870922	<i>yvrL</i> -r	3410873-3410897	<i>gerAA</i> -f [*]	3390035-3390058
<i>cstA</i> -f	2936841-2936823	<i>racE</i> -f	2902930-2902954	<i>ysx</i> -f	2869868-2869845	<i>yvrK</i> -f	3409810-3409828	<i>yuxN</i> -r	3387834-3387810
<i>cstA</i> -r	2936391-2936409	<i>racE</i> -r	2902641-2902664	<i>ysx</i> -r	2869529-2869552	<i>yvrK</i> -r	3410204-3410186	<i>yuxN</i> -f	3387490-3387515
<i>ysfA</i> -r	2930958-2930935	<i>rpb</i> -f	2900815-2900791	<i>spolIB</i> -f	2863798-2863174	<i>yvrI</i> -f	3409021-3409003	<i>yvgB</i> -f	3386039-3386021
<i>ysfA</i> -f [*]	2930827-2930851	<i>rpb</i> -r	2900546-2900569	<i>spolIB</i> -r	2862791-2862815	<i>yvrI</i> -r	3408766-3408784	<i>yvgB</i> -r	3385597-3385615
<i>ysfB</i> -r	2931835-2931813	<i>ysnA</i> -r	2899771-2899795	<i>maf</i> -f	2862238-2862214	<i>yvrH</i> -f [*]	3408426-3408408	<i>yvgA</i> -f	3384930-3384948
<i>ysfB</i> -f	2931429-2931451	<i>ysnA</i> -f	2899996-2899972	<i>maf</i> -r	2861937-2861961	<i>yvrH</i> -r	3407976-3407994	<i>yvgA</i> -r	3385150-3385132
<i>ysfC</i> -r	2933117-2933093	<i>ysnB</i> -f	2899529-2899505	<i>ysx</i> -f	2861650-2861626				
<i>ysfC</i> -f	2932778-2932802	<i>ysnB</i> -r	2899215-2899239	<i>ysx</i> -r	2861334-2861358				
<i>ysfD</i> -r	2934575-2934553	<i>ysnF</i> -r	2898837-2898816	<i>ysx</i> -B	2854758-2854736				
<i>ysfD</i> -f	2934181-2934203	<i>ysnF</i> -f	2898557-2898582	<i>ysx</i> -B	2854459-2854482				
<i>ysgA</i> -f	2930576-2930552	<i>ysnE</i> -r	2897745-2897722	<i>rplU</i> -f	2854709-2854688				
<i>ysgA</i> -r	2930440-2930464	<i>ysnE</i> -f	2897475-2897496	<i>rplU</i> -r	2854503-2854524				
<i>ysgB</i> -f	2925738-2925717	<i>ysnD</i> -r	2897167-2897143	<i>rpmA</i> -f	2854364-2854341				
<i>ysgB</i> -r	2925412-2925434	<i>ysnD</i> -f	2896902-2896926	<i>rpmA</i> -r	2854218-2854242				
<i>ysbA</i> -f [*]	2925011-2924987	<i>ilvB</i> -f	2895670-2895626						
<i>ysbA</i> -r	2924810-2924835	<i>ilvB</i> -r	2894859-2894882						

* Transcripts not detected by RT-PCR.

criptional analysis. Previous experiments carried out in our laboratory (A. Hernández, V. Parro & R. P. Mellado, unpublished) demonstrated that RT-PCR analysis permitted detection of transcription of almost twice as many genes as Northern blot analysis; we therefore explored the possible use of RT-PCR for global transcriptional detection and monitoring of gene expression.

As a way to scale up the RT-PCR reaction, amplification of total RNA preparations with different combinations of primer oligonucleotide pairs was assayed. The oligonucleotide pairs *lytT*-f/*lytT*-r, *lytS*-f/*lytS*-r and

yslB-f/*yslB*-r (Table 1), corresponding to genes *lytT*, *lytS* and *ystB*, respectively, were used as primers for RT-PCR reactions. The RT-PCRs were carried out in the presence of 500 ng total RNA, either with each pair of primers individually, or with combinations of two or three pairs. A reaction without reverse transcriptase was carried out as a negative control in all cases to ensure that the resulting DNA fragment was not due to amplification of contaminant DNA (not shown). The results proved that combined RT-PCRs for more than one gene in the same reaction resulted in competition for the enzyme with subsequent loss of the corresponding bands. Since *lytS* and *lytT* may form a potential two-

gene operon, as deduced from the genome sequencing data (Kunst *et al.*, 1997), the results obtained also illustrate that different pairs of primers for the same transcript could also compete in the combined RT-PCR analysis.

To overcome these difficulties, RT-PCR analysis for individual genes was performed. Fig. 1 shows representative results obtained when putative transcripts for some of the newly found genes in the five different regions of the *B. subtilis* genome were amplified by RT-PCR using as a template total RNA isolated from bacterial cultures grown in minimal medium to mid-exponential phase. All the products obtained were of the expected size and the absence of product in the reactions without RT demonstrates that the bands observed with reverse transcriptase derived from RNA and not from possible contaminant DNA. Most of the genes in all chromosomal regions tested had transcripts which were amplified by RT-PCR when 500 ng total RNA were used in the reaction (as in Fig. 1); increasing the amount of RNA template to 1 µg or adding amplification cycles to the reaction did not alter the results (not shown). Genes that did not have RT-PCR-detectable transcripts are shown with an asterisk in Table 1 and amounted to only 18.7% of the genes tested.

Monitoring of differential gene expression

We also tested whether the RT-PCR technique could be used to distinguish individual genes that are differentially expressed depending on environmental and/or physiological conditions. The presence of the *spoVID* transcript was monitored by RT-PCR amplification using 500 ng total RNA preparations from bacteria induced to sporulate by nutrient exhaustion in Schaeffer's medium (Harwood & Cutting, 1990) in the presence of the appropriate pair of primers (Table 1). Expression of *spoVID* is known to peak at about the second hour of sporulation and continue throughout spore development (Beall *et al.*, 1993). The results of these experiments are shown in Fig. 2(a). The gene is expressed more abundantly during sporulation than in exponential phases of growth; the different amount of amplified material after the RT-PCR reaction in each case reflected the expected difference in the relative expression level (Beall *et al.*, 1993) (the transcription detected before T_2 probably reflects the natural asynchrony of the culture), clearly showing that RT-PCR amplification can also be of use to distinguish between different levels of gene expression. Transcription from the *ara* operon is not detected when bacterial cells are grown in the absence of arabinose. When cells were grown in the presence of arabinose, the operon became induced (Sá-Nogueira & Ramos, 1997) and transcription from two genes of the operon (*abfA* and *araL*) was clearly detected by RT-PCR (Fig. 2b). These results indicate that some of the silent ORFs in the *B. subtilis* genome might not be expressed unless properly induced, as is the case with the *ara* operon, and that RT-PCR could be used to detect differential transcription of genes.

Fig. 1. RT-PCR analysis for individual genes. Reactions were carried out using the corresponding pairs of oligonucleotide primers for the indicated genes. + and - indicate lanes where the reverse transcriptase was present or absent in the reaction, respectively. Total RNA was from cells grown to mid-exponential phase in minimal medium. (a) Genes from 2744 to 2767 kb, (b, c) genes from 2854.4 to 2964.4 kb, (d) genes from 3384 to 3430 kb, (e, f) genes from 936.6 to 976.6 kb, (g) genes from 1265.4 to 1280 kb. Sizes of the amplified fragments ranged between 123 bp (*yjdJ*) and 601 bp (*adhB*).

RT-PCR sensitivity

To monitor the sensitivity of the RT-PCR reaction for the detection of transcripts of poorly expressed genes versus those with a higher expression, serial dilutions of

Fig. 2. Differential gene expression. Reactions were carried out using the corresponding pairs of oligonucleotide primers for *spoVID* (a) or *abfA* and *araL* (b). + and - indicate the lanes where reverse transcriptase was present or absent in the reaction, respectively. (a) Total RNA from cells growing in sporulation medium at exponential (lane 1), late exponential (lane 2) and sporulation (lanes T_0 – T_8) phases. (b) Total RNA from cells grown in the presence (lanes 1, 2, 5 and 6) or absence (lanes 3, 4, 7 and 8) of arabinose was used as a template for the reactions carried out using the *abfA* pair of primers (lanes 1–4) or the *araL* pair of primers (lanes 5–8). Labels on the left indicate the sizes of the amplified fragments in bp.

Fig. 3. Sensitivity of the RT-PCR. Reactions were carried out using the corresponding pairs of oligonucleotide primers for *ygaI* (a) or *ygaD* (b). Serial dilutions of a total RNA preparation from cells grown in minimal medium were used as templates for the different reactions: 500 ng (lane 1), 250 ng (lane 2), 100 ng (lane 3), 50 ng (lane 4), 10 ng (lane 5), 5 ng (lane 6) and 1 ng (lane 7). In a separate reaction, 500 ng total RNA was incubated in the absence of reverse transcriptase (lane 8) as a control. Labels on the left indicate the sizes of the amplified fragments in bp.

total RNA preparations were used as templates for the RT-PCR amplification of mRNAs from a gene, the transcript of which was detected by Northern blot

analysis (*ygaI*; A. Hernández, V. Parro & R. P. Mellado, unpublished results) and from another gene, the transcript of which was only detected by RT-PCR analysis (*ygaD*). Fig. 3(a) shows that transcription from *ygaI* could be detected even when only 5 ng total RNA was used as a template, whereas 50 ng total RNA seemed to be the minimum amount needed to clearly detect the *ygaD* transcript (Fig. 3b). Therefore, a 10-fold difference in the level of detection could be established between the two kinds of transcripts. Considering that a maximum of 5 % of a total RNA preparation is mRNA and that the *B. subtilis* genome contains approximately 4000 protein-encoding genes (Kunst *et al.*, 1997), and assuming that all genes are transcribed (at least to an extent detectable by RT-PCR) and a mean yield of about 3 mg RNA per ml of culture that contains 2×10^9 cells, the RT-PCR detection limit appears to be approximately 0.7 (less than one) transcripts per cell. Therefore the RT-PCR approach is more sensitive than the conventional Northern blot analysis and appears to be more sensitive than the assay based on total RNA hybridization to oligonucleotide arrays (de Sazieu *et al.*, 1997), where detection limits were reported to be around two transcripts per cell.

From the results obtained it is concluded that RT-PCR could be a useful technique for simultaneous transcriptional analysis of large regions of bacterial genomes since it is easy to use, does not require hybridization to labelled probes, is very sensitive, differentiates between expression levels, and can be used for the analysis of coordinately expressed genes.

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