Background

Methods for predicting protein-coding genes have been extensively optimized [1], while the development of methodology for the structural and functional analysis of intergenic regions has only come into focus more recently.

Although the fraction of non-coding and/or intergenic sequences in prokaryotic genomes is relatively low compared to eukaryotes [2], it encompasses a similar complexity in conserved DNA sequence motifs and regulatory elements [3]. In prokaryotes, elements such as binding sites for the sigma factor unit of the RNA-polymerase [4] are essential for appropriate guidance of the transcription machinery. Sigma factors enable the binding of the RNA polymerase core enzyme to the canonical promoter sequences they recognize. Various sigma factors have been identified and many bacteria possess more than one sigma factor [5], each recognizing specific DNA sequence motifs in promoters, which enables cells to respond rapidly to changing conditions by sigma factor-mediated adjustment of gene transcription patterns. Although different sigma factors recognize specific promoter sequences, the target sequence elements recognized by particular classes of sigma factors have been reported to be conserved among bacteria [4,6]. For example, $\sigma^{34}$ [7,8] and $\sigma^{14}$ [9] factors were shown to recognize similar sequences in phylogenetically distant bacterial species. Further fine-tuning of transcription activity by the sigma-factor containing RNA polymerase (e.g., harbouring the canonical $\sigma^{70}$) involves regulatory factors that either repress or activate transcription by binding to specific DNA sequence motifs...
Prediction of conserved motifs in the intergenic regions of *L. plantarum*

With 3.3 million nucleotides, the genome of *L. plantarum* WCFS1 is the largest *Lactobacillus* genome and comprises 3137 predicted genes, of which 3051 are protein-encoding (Refseq NC_004567, [23]). The intergenic regions encompass approximately 16 percent of the entire genome nucleotide sequence (530640 out of 3308275 nucleotides). Using the genome annotation of *L. plantarum* WCFS1, 2209 intergenic sequence upstream of protein-encoding genes within the genome of *L. plantarum* WCFS1, *In silico* models of recognizable σ^70^-promoter sequence elements were generated using position weight matrices (PWM; [19,20]), which were subsequently used for genome-wide prediction of promoter sites. Using this approach we predicted 3874 candidate σ^70^-promoters in the *L. plantarum* WCFS1 genome at p-value cut-offs of 10^-4_. Subsequently, transcriptome datasets obtained using strand-specific genome-wide tiling DNA microarrays were used to validate the transcriptionally active proportion of the in silico predicted σ^70^-promoter sequence elements [12,21,22], enabling the validation of 568 of the predicted promoters by their proximity (≤40 nucleotides) to 1167 transcription start sites inferred from experimentally estimated transcript boundaries.

**Detection of putative transcription start sites**

As described in previous studies, the high sensitivity of *in silico* prediction of σ^70^-promoters using PWMs usually generates many false-positive identifications [3,28–30]. To overcome this limitation, putative transcription start sites (TSSs) inferred from 5'-ends of transcripts are commonly employed to corroborate predicted σ^70^-promoter locations [12,21,31]. In this study, we determined genome-wide putative TSSs from tiling-array derived transcription data obtained for *L. plantarum* WCFS1 grown in rich laboratory medium (MRS) at 37°C. Transcriptional activity was assigned to genomic regions by employing a global threshold approach [32], leading to the identification of 1167 distinct transcriptionally active regions (TARs) larger than 120 nucleotides [33]. The 1167 TARs cover 1995 of the 3137 predicted genes in the *L. plantarum* WCFS1 genome, likely reflecting the transcriptionally active fraction of the entire genomic coding capacity under the growth conditions employed in this study.

The 5'-ends of TARs may be the result of transcription initiation but can also arise from other events, such as degradation, processing or cross-hybridisation. Although in the majority of cases 5'-end points of TARs are probably the TSSs [12] false-positive identifications are possible (see Results S2, Figure S2 and S3). Inspection of signal strength patterns at the 5'-end of different TARs showed that revealed sharper increases in signal intensity for highly expressed TARs compared to weakly expressed TARs. Consequently, the assignment of putative TSSs to weakly expressed TARs leads to more ambiguous TSS positioning as compared to those assigned to highly expressed TARs. Therefore, TSSs were grouped according to their mean signal intensity (see Methods), which allowed the discrimination between TSSs assigned to highly and weakly expressed TARs. To consistently assign TSSs to TARs an algorithm was developed that identifies the maximum increase
in signal intensity at the 5’-end of a TAR and by linear extrapolation of the local transcription signal-intensity slope estimates the position of the TSS of the TAR. We evaluated the performance of our TSS predictions by correlating the tiling array-based TSSs with those that were previously identified in *L. plantarum* by primer extension. For nearly all cases (11 out of 12 genes) the putative TSS predicted on basis of the tiling array data was within a maximum distance of 28 nt from the experimentally TSS determined by primer extension (see Results S3). The majority of these genes (8 genes) were highly expressed in the tiling array dataset (their average expression signal belonged to the two groups of TARs that showed the highest expression levels, see Method).

Application of the algorithm allowed prediction of a TSS for each of the 1167 TARs. Expectedly, out of the 1167 predicted TSSs, the majority were located upstream of protein encoding genes (912). Next to these expected TSSs, 149 of the predicted TSSs appeared to be positioned in an antisense orientation relative to the annotated genes, while an additional 106 predicted TSSs were positioned in genomic regions that lack genetically linked genes, suggesting that these TSSs could represent the start of non-coding RNAs (see Figure 2).

**Figure 1.** **Highly conserved motifs.** Highly conserved motifs (E-value<10^{-80}) detected by MEME search in the upstream regions of protein-encoding genes (for a full list see Figure S1 in Supplementary results). Motifs are shown as Seqlogos [45].

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<table>
<thead>
<tr>
<th>Motif Description</th>
<th>E-value</th>
<th>Seqlogo representation</th>
</tr>
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<tbody>
<tr>
<td>1 Shine-Dalgarno sequence</td>
<td>2.7x10^{-29}</td>
<td><img src="image1" alt="Seqlogo" /></td>
</tr>
<tr>
<td>2 Extended -10 promoter</td>
<td>1.1x10^{-19}</td>
<td><img src="image2" alt="Seqlogo" /></td>
</tr>
<tr>
<td>3 -35 -10 promoter</td>
<td>3.8x10^{-82}</td>
<td><img src="image3" alt="Seqlogo" /></td>
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and their relative distance to predicted promoters can be used to corroborate the involvement of these promoters in the initiation of transcription of the corresponding TAR. The TSS locations were correlated to appropriately oriented predicted σ^70-promoters (p-value < 10^{-3}) in close proximity of the TSS, identifying 568 σ^70-promoters within a distance 40 nt of a TSS (see Figure 2) of which 474 σ^70-promoters were within a distance 20 nt of a TSS (tiling-array resolution 14 nt) (see Figure 3). About 70% of TSSs assigned to highly expressed TARs (group A and B in Figure 4) were in close proximity of a predicted σ^70-promoter (≤40 nt), while for ‘only’ 17% of TSSs detected for low expressed TARs (group E in Figure 4) a σ^70-promoter could be assigned. Expectedly, TSSs inferred from expression signals of highly expressed genes give better corroboration results than TSSs inferred from expression signals of lower expressed genes. The lower promoter assignment to the low-expression TARs may be due to the ambiguity of the TSS prediction for these TARs, while the higher accuracy of TSS prediction for high expression TARs provides more confident co-

**Figure 2. Flow chart of promoter validation.** Genome-wide motif search using MEME and MAST provided 3874 and 14962 predicted σ^70-promoters at p-value cut-offs of 10^{-4} and (10^{-4}≤p-value<10^{-3}), respectively. For validation of the predicted promoters, transcriptionally active regions (TARs) were detected using tiling array data of *L. plantarum* WCFS1 and for each TAR the transcription start site (TSS) was determined. After filtering the TSSs for low-expressed TARs, 621 TSSs were obtained for validation of predicted σ^70-promoters. At a low p-value cut-off (p-value<10^{-4}), 568 σ^70-promoters were found that co-localized within a distance of 40 nt of TSSs. An additional 120 σ^70-promoters were found to co-localized within a distance of 40 nt of TSSs after raising the p-value cut-off (10^{-4}≤p-value<10^{-3}). For 94 TSSs, no co-localized σ^70-promoter (within a distance of 40 nt) was found.

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localizations with corresponding promoter predictions. Overall, TSSs and predicted promoters displayed significant co-occurrence (Fisher exact p-value < 10^-6), based on the detected TSSs matching with predicted promoters in comparison with randomly generated TSSs. The expected co-occurrence frequencies of TSSs and promoters within a range of 40 nucleotides are predicted to be 4% (see Figure 4, group Random).

As σ^70 target sequences may exhibit quite extensive sequence variation, this approach may have missed recognizable, but less conserved, σ^70-promoter sequence elements. To expand our analyses to also encompass σ^70-promoters with more deviating sequences, the stringency of promoter detection was relieved (10^-3 < p-value < 10^-5), allowing MAST-based detection of an additional 14962 sequence elements with σ^70-promoters similarity. Of these predicted elements 276 co-localized within a distance of 40 nt of TSSs for which no conserved σ^70 promoters (p-value < 10^-6) could be found (see Figure 2 and Figure S4). However, TSSs and these lower stringency predicted promoters displayed no significant co-occurrence (Fisher exact p-value = 0.16) and co-occurrence frequencies of TSSs and promoters within a range of 40 nucleotides are predicted to be 19% (see Figure S5).

Discussion

In this study we determined the consensus motifs of the σ^70 promoters in L. plantarum WCFS1 using different models and approaches, including the validation of predicted promoters using tiling array transcriptome datasets.

Searching for conserved motifs in the intergenic regions of L. plantarum WCFS1 revealed highly conserved motifs (see Figure 1). Analogous to their anticipated sequence conservation, the two elements of the general σ^70 promoter, the so-called –35 and –10 elements, were among the most conserved motifs (p-values < 10^-4).

![Figure 3. Distribution of distances between promoters and TSSs. Distribution of distances (in nt) for 568 cases where a predicted σ^70-promoter (genome-wide search, p-values < 10^-4) and a TSS were found in proximity (≤60 nt) of each other. TSSs are divided into 5 groups (A, B, C, D, E) defined by the differences between the means of signal intensities upstream and downstream of the TSS (see Methods).](image)

![Figure 4. Frequency distribution of validated promoters. Frequency distribution of σ^70-promoters (genome-wide search, p-values < 10^-5) with TSSs found in proximity (≤40 nt) of each other. Frequencies are given for 5 groups (A, B, C, D, E) and the expected frequency of co-localized TSSs and promoters (Random). The 5 groups are the inter-percentile ranges (E: 0–20%, D: 21–40%, C:41–60%, B: 61–80%, A: 81–100%) of the ranked mean differences between the means of signal intensities upstream and downstream of the TSS (see Methods).](image)

The σ^70 promoter related motifs were used to generate position weight matrix (PWM) based models. PWM models enabled the genome-wide detection of 3874 and additional 14962 putative σ^70 promoters in the L. plantarum WCFS1 genome at p-value cut-offs of 10^-4 and 10^-3, respectively. Removing all σ^70 promoters that were intergenic but overlapped partly with the 5’- or 3’-ends of genes yielded 1763 and 4093 σ^70 promoters at p-value cut-offs of 10^-4 and 10^-3, respectively. As shown in previous studies on lactic acid bacteria [16], the average number of transcriptional units is about 1077 with an average of 1.77 genes per unit suggesting that the number of σ^70 promoters reflects the number of transcripts that would be expected on basis of the number of predicted operons on the genome of L. plantarum [16]. The additional σ^70 promoters partly overlapping with coding parts of genes might be alternative σ^70 promoters (see for example Results S2) which are also found in other bacterial genomes [34].

PWMs have been used to predict σ^70-promoters with high sensitivity in other species [3,35,36], but were hampered by many false-positive identifications [3,28–30], illustrating the difficulty to model the general σ^70-promoter sequence elements, which are bipartite motifs composed of 2 conserved sequence motifs separated by a non-conserved spacer sequences of variable length [27,37,38].

Analogous to what was described in previous studies [12,21,31], transcription start sites (TSS), determined by high-resolution tiling arrays, can be used to corroborate predicted σ^70-promoter locations. The analysis of transcriptome datasets from L. plantarum WCFS1 obtained with strand-specific genome-wide tiling arrays revealed 1167 transcriptionally active regions (TARs). The 5’-ends of TARs were used to infer putative TSSs (see Figure 2). To confirm the TSSs inferred from the 5’-ends of TARs these TSSs were compared to TSSs that have previously been determined by primer extension analysis (see Results S3). Our predicted TSSs co-localised nicely with the previously described TSS positions. With the available TSS information, we were able to show that our approach gives highly reliable results. Similarly, alternative...
genome wide transcription analysis methods such as RNA-seq can also be employed to detect TSSs in bacteria [31,39,40], but here we confirm that high-resolution tiling array data are a valuable resource to infer TSS locations [12,21,22].

Of the 1167 putative TSSs selected for validation, 568 TSSs were positioned within proximity of the $\sigma^{70}$ promoters that were predicted with high stringency, while an additional 276 TSSs could be co-localized with predicted promoters using the lower stringency promoter prediction ($10^{-4}$-p-values$<10^{-5}$). However, the latter co-occurrence of TSSs and low-stringency predicted promoters was not significant (Fisher exact p-value $= 0.16$), which is likely due to a substantial overprediction (false-positives) of promoter sequences.

In general, promoters function more efficiently with sequence elements that resemble the consensus sequences. However, most promoters have non-consensus sequences and their activity is further modulated by trans-acting factors (including alternative sigma factors, small ligands and transcription factors) [33]. To discover conserved transcription initiation motifs other than the $\sigma^{70}$ promoter-related sequences, DNA sequences upstream to TSSs with no $\sigma^{70}$ promoter were searched for other conserved motifs (applying MEME searches as described for the initial search, see Method). These searches did not reveal additional highly-conserved motifs (p-value cut-off$<10^{-4}$; data not shown).

Our results show that motif-based approaches can be used to recover conserved sequence elements of the $\sigma^{70}$ promoter. However, the discovery of more degenerate promoter elements is hampered by many false-positive identifications [3,28-30] and validation of predicted promoters (e.g. using transcriptome data) is required but limited by the statistical significance of the obtained results. Our findings suggest that more degenerate $\sigma^{70}$ promoters may constitute a considerable proportion of all $\sigma^{70}$ promoters in a given genome (in our case about 24% of the validated $\sigma^{70}$ promoters have p-values$\leq 10^{-4}$), which has been described for other bacterial genomes (such as E. coli) [34]. In this study, transcription data under a single growth condition was used limiting the validation to the active portion of $\sigma^{70}$ promoter-related genes that are clearly transcribed. Transcription data obtained under a variety of growth conditions would still be required to validate the full complement of functional $\sigma^{70}$ promoters and distinguish them from false-positive predictions.

Materials and Methods

Transcription analysis of Lactobacillus plantarum WCFS1

For genome wide transcriptome analysis L. plantarum WCFS1 [18] was grown until mid-exponential growth phase in MRS, cells were harvested and RNA was isolated and labeled as described before [41,42]. 1.5 $\mu$g of labeled targets were hybridized and analyzed on WCFS1 DNA microarrays that cover the whole genome in a tiling fashion as described (GSE36898, GPL15385, GPL15386). The gene expression data are MIAME compliant and deposited in the gene expression omnibus (GEO) database (accession numbers GSE36898, GPL15385, GPL15386).

The hybridization intensities of two biological replicates were obtained. Hybridization intensities were log2-transformed, background corrected and normalized by quantile normalization [43]. After normalization, intensities of the two replicates were averaged yielding two strand-specific transcript-level data sets.

Building $\sigma^{70}$-promoter models using PWMs

Position weight matrices were constructed from high-scoring motifs identified by MEME [24] searches on a selected subset of intergenic DNA sequences of the L. plantarum WCFS1 genome.

Initially, all 100 nt regions upstream of all protein encoding genes were selected from the L. plantarum WCFS1 genome (Refseq NC_004567). Subsequently, these sequences were evaluated for their potential overlap with a preceding gene, and in such cases, only the intergenic sequence was used for analysis, provided they were 25 nt or longer.

The resulting set of selected intergenic sequences was searched for conserved motifs using MEME [24], applying standard DNA parameter settings. Only motifs reported by MEME with E-values below $10^{-4}$ were considered relevant for further analysis. Additional parameters used for selection of candidate sequences were: 1) Zero or One Occurrence Per Sequence (ZOOPS mode), 2) a maximum of ten different motifs per sequence, and 3) each motif should be found in at least thirty-five different sequences. PWM models were constructed for the most abundantly encountered motifs, including those resembling the canonical $-35$ and $-10$ elements known from general $\sigma^{70}$-dependent promoters [6].

Predicting $\sigma^{70}$ promoters

The PWM models were used in a MAST [26] search against the complete genomic sequence of L. plantarum WCFS1 including the coding regions to predict potential $\sigma^{70}$ promoters. MAST hits with relatively high significance (p-values$<10^{-7}$) were used in further analysis, unless indicated otherwise.

Because the spacer region between the $-35$ and $-10$ boxes of the $\sigma^{70}$-promoter varies in length (16-20 nt) [27], PWM models were manually altered by either copying or deleting the columns in the position weight matrix that were part of the spacer region and had the least functional information (low overall specificity and low sequence conservation) of this region. These new matrices were used in genome-wide MAST searches to predict additional promoter occurrences with different spacer-lengths. All predicted promoters (including those predicted on basis of the extended $-10$ box motif) were filtered for overlapping promoter elements. In case of overlap, the hit with the highest significance was used for further analysis. All non-intergenic promoter elements were removed.

Detecting transcriptionally active regions

A global threshold approach was used to detect significantly transcribed genomic regions. For each strand-specific dataset the global threshold was determined from the log distribution of the probe signals. A background normal distribution was estimated from the left-of-the-mode data [43] and used to estimate the background probability of a probe signal. The global threshold was set to a signal level at which the probability of a probe signal being a background signal was less than $10^{-5}$. Next, transcriptionally active regions (TARs; [33]) were determined by applying a segmentation algorithm (comparable to transfrag as described in [32] and [44]) using the previously estimated global threshold. Segments of adjacent probes with signal intensities above the threshold were identified. Adjacent segments with a gap of maximal 60 nt (corresponding to the length of a single probe on the tiled micro-array) were joined and, segments shorter than 120 nt were discarded.

Transcription start site determination and statistical analysis

The 5’-end of a TAR was used to estimate the corresponding TSS position. Manual inspection of signal patterns of different TARs in the tiling array data revealed that highly expressed regions display a rather sharp, approximately linear rise in signal intensity while in lowly expressed regions the signal change...
appeared to be sigmoidal (data not shown). Based on this observation, we developed the following procedure to determine TSS: Probe signals from the 5' end of a TAR and its upstream region were used to determine the genomic position at which the slope of increase in signal intensities reached its maximum. This position was used to linearly extrapolate the position of the TSS using the difference between the probe signal of the maximum slope position and the background signal (derived from the mode of the distribution of the probe signals).

Using the gene annotation of *L. plantarum* WCFS1 (Refseq NC_004567, [23]), TSSs were labelled anti sense if the transcribed strand (TAR) was opposite to a gene-containing strand. In addition, if a TAR had no overlap with known genes (based on the current gene annotation) the assigned TSS and its corresponding TAR were annotated as non-coding. All other TSSs were assigned to the closest downstream location gene positioned on the same strand.

To estimate the probability for randomly selecting a TSS in a 40 nucleotide range from a promoter, we used the genomic positions of the TSSs, randomly selected genomics positions of promoters (10,000 times) and calculated for each TSS the minimum distance to the 3' end of a promoter.

TSSs were grouped according to the increase in signal intensity by (1) calculating the differences between the means of expression signal upstream and downstream of the TSS and (2) division of the TSS into five groups according to their rank position (inter-percentile ranges 0–20%, 21–40%, 41–60%, 61–80% and 81–100%).

**Supporting Information**

**Results S1** Conserved motifs detected by MEME in the upstream regions of protein-encoding genes in *L. plantarum* WCFS1. (DOCX)

**Results S2** Interpretation of TAR detection results. (DOCX)

**Results S3** Evaluation of results of the TSS prediction method. (DOCX)

**Figure S1** Motifs detected by MEME in *L. plantarum* WCFS1. Motifs are displayed as Seqlogo’s that contain at each position stacks of letters. The height of an individual letter in a stack represents the probability of the letter at that position in an occurrence of the motif. The position-specific probabilities are retrieved from the position-specific weight matrices calculated by MEME. (TIF)

**Figure S2** Inferred signal intensity of a genomic region containing two genes. Inferred signal intensity plotted on part of the *L. plantarum* WCFS1 chromosome (genome location: 1156200–1159500 of the forward strand). At this location two genes are located (*lp_1273* and *lp_1274*) which are considered to form an operon. The tiling microarray signal intensities measured confirms that both genes are transcribed at an approximately equal level and suggest that the inferred transcription start is 537 nucleotides upstream of *lp_1273*. The black box indicates the position of a predicted σ70-promoter. The grey box indicates the position of a second predicted σ70-promoter with no close TSS. (TIF)

**Figure S3** Inferred signal intensity of a genomic region containing two genes. Inferred signal intensity plotted on part of the *L. plantarum* WCFS1 chromosome (genome location: 1407149–1410217 of the forward strand). At this location two genes are located (*lp_1541* and *lp_1543*) expressed at different levels. For *lp_1541* a putative TSS and a predicted σ70-promoter co-localized (2 nt apart) upstream to the annotated translation start of the gene. For *lp_1543* a putative TSS is located 145 nt downstream of the annotated translation start of *lp_1543* and a σ70-promoter is predicted 31 nt upstream of this translation start. The low signal expression of *lp_1543* makes it difficult to infer a clear TSS position. The black boxes indicate the positions of predicted σ70-promoters. (TIF)

**Figure S4** Distribution of distances between promoters and TSSs. Distribution of distances (in nt) for 568 cases where a predicted σ70-promoter (10−4<p-values<10−3) and a TSS were found in proximity (≤60 nt) of each other. TSSs are divided into 5 groups (A, B, C, D, E) defined by the differences between the means of signal intensities upstream and downstream of the TSS. The 5 groups are the inter-percentile ranges (E: 0–20%, D: 21–40%, C: 41–60%, B: 61–80%, A: 81–100%) of the ranked mean differences (see Methods). At distances above 40 nt the observed number of co-localized TSSs and promoters (≤10 counts) is equal to the expected number co-localized TSSs and promoters (see Methods). (TIF)

**Figure S5** Frequency distribution of validated promoters. Frequency distribution of σ70-promoters (10−3≤p-values<10−2) with TSSs found in proximity (≤40 nt) of each other. Frequencies are given for 5 groups (A, B, C, D, E) and the expected frequency of co-localized TSSs and promoters (Random). The 5 groups are the inter-percentile ranges (E: 0–20%, D: 21–40%, C: 41–60%, B: 61–80%, A: 81–100%) of the ranked mean differences between the means of signal intensities upstream and downstream of the TSS (see Methods). (TIF)

**Author Contributions**

Conceived and designed the experiments: MK. Performed the experiments: RSB. Analyzed the data: TJT MW SvH. Contributed reagents/materials/analysis tools: RSB MW TJT. Wrote the paper: TJT MW SvH.

**References**