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RESEARCH ARTICLE

Identification of a mitotic recombination hotspot on chromosome III of the asexual fungus *Aspergillus niger* and its possible correlation elevated basal transcription

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Abstract Genetic recombination is an important tool in strain breeding in many organisms. We studied the possibilities of mitotic recombination in strain breeding of the asexual fungus *Aspergillus niger*. By identifying genes that complemented mapped auxotrophic mutations, the physical map was compared to the genetic map of chromosome III using the genome sequence. In a program to construct a chromosome III-specific marker strain by selecting mitotic crossing-over in diploids, a mitotic recombination hotspot was identified. Analysis of the mitotic recombination hotspot revealed some physical features, elevated basal transcription and a possible correlation with purine stretches.

Keywords Parasexual cycle · Physical map · Somatic cross · Genome atlas · Purine stretch · Transcription

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Introduction

Aspergillus niger fermentation products have many uses in food applications, for example, as a producer of organic acids and enzymes (Archer 2000). Production processes have been thoroughly optimized by improvements in process technology (Naidu and Panda 1998; Schügerl 2000) and by strain breeding (Punt et al. 2002). Classical improved strains have been obtained using random mutagenesis, recombination and genetic modification based on transformation of protoplasts.

Because of its haploid nature, *A. niger* production strains can be improved by subsequent rounds of mutation and selection. Genetic recombination is more troublesome because *A. niger* lacks a sexual cycle. An alternative is recombination using the parasexual cycle, which first was described for *Aspergillus nidulans* (Pontecorvo et al. 1953). The parasexual cycle starts with a heterokaryon, which can be obtained via anastomosis or protoplast fusion. Using double markers, a heterokaryon can be selected and maintained on synthetic medium. A heterozygous diploid, formed by the fusion of the two different nuclei, can be selected as a prototroph. Through chemically induced loss of chromosomes, this diploid is reduced to an unstable aneuploid, finally giving rise to stable haploids. Essentially in this way complete chromosomes are exchanged between the starting strains. Markers with less than 25% recombination are believed to be located on the same chromosome. Using the parasexual cycle, 89 genetic markers were assigned to a linkage group and several marker strains were constructed (Bos et al. 1993).

Although in *A. niger* mitotic crossing-over of homologous chromosomes occurs at low frequencies, it is the only method used to determine the gene order on a linkage group. In a heterozygote diploid, all markers distal to a

cross-over become homozygous. Debets et al. (1993) used several genetic markers to select such partially homozygous diploids, thereby constructing a genetic map of *A. niger* which shows the gene order of 60 markers.

For the improvement of strain properties, such as enzyme production, the construction of a diploid may result in improved isolates (Khattab and Bazaraa 2005; Loera and Cordova 2003; Montiel-Gonzalez et al. 2002). By completing the parasexual cross, also recombinants with higher enzyme production than the parental strains have been isolated (Ball et al. 1978; Das 1980; Das and Ghosh 1989). This strategy was also successfully used for genetically modified production strains (Bodie et al. 1994). Furthermore, the parasexual cycle can be used for recombination of less compatible strains via protoplast fusion. Heterokaryons that would not be formed otherwise can be obtained this way, and haploidization followed by selection has resulted in the isolation of improved strains (Rubinder et al. 2000).

Usually recombinants are selected randomly, after which their performance is compared to their parents. By first assigning mutations or disruptions to a linkage group, recombination of unlinked properties can be achieved in an orchestrated manner (Swart et al. 1990; van den Hombergh et al. 1997).

Recombination of linked mutations is troublesome because of the low rate of mitotic crossing-over in *A. niger*. Meiotic crossing between mitotically linked markers is much more frequent and provides a means to establish reliable genetic maps. Possibly an increased rate of mitotic crossing-over could approach the advantages of meiotic recombination. In several organisms, mitotic crossing-over has been induced using heat shock, chemicals or irradiation (Becker et al. 2003; Davies et al. 1975; Hilton et al. 1985; Jansen 1964; Klinner et al. 1984; Sermonti and Morpurgo 1959; Whelan et al. 1980). In order to examine the use of increasing mitotic recombination with effectors in *A. niger*, a chromosome specific tester strain would be very useful.

With the *A. niger* genomic sequence available (Pel et al. 2007), the genetic map can be improved by cloning a number of markers, thus linking the genetic map to the physical map. Using such linked markers, we investigated mitotic crossing-over via selection of partially homozygous diploids. For this purpose we chose to study markers on chromosome III for several reasons; many markers are available on both arms, on the left arm the cloned markers *pyrA* and *areA* are present and in parasexual crosses the linked markers *bioA1* and *lysA7* were found to have recombination frequencies around 15% (Bos et al. 1988). Furthermore, the sequence information for chromosome III, located on supercontigs 12 and 15 adds up to 4.5 Mb (Pel et al. 2007), while the estimated size by CHEF gels is 4.1 Mb (Verdoes et al. 1994a). This indicates that the sequence information for this chromosome must be close to complete. Using the improved genetic map of chromosome III, we constructed specific marker strains with which we studied the occurrence and frequency of mitotic crossing-over.

Materials and methods

The *A. niger* strains used were derived from CBS 120.49 and were kindly provided by the Laboratory of Genetics, Wageningen University, The Netherlands. The strains used to construct diploids and new master strains are listed in Table 1. Strains used for the complementation of genetic markers are N495 (*adeB2; nicA1*) for *adeB*, N521 (*fwnA1; adeE8*) for *adeE8*, N658 (*lysA7 argD6; nicA1*) for *argD* and *lysA*, N660 (*bioA1 argL2; nicA1; pabA1*) for *argL*, N687 (*fwnA1; proC3 lysA7 cysA2*) for *cysA* and *lysA*, N705 (*fwnA1; cnxD6; leuA1; nicA1*) for *cnxD*, N733 (*hisD4; adeG13*) for *adeG13*, N884 (*bioA1 lysE28)* for *bioA* and *lysE*, N885 (*bioA1 hisH8; nicB5*) for *hisH*, N901 (*olvA1; bioB2; argI15 metB10*) for *bioB*. All strains carry the *cspA1* mutation, conferring low conidiophores and dense sporulation.

PCR cloning and complementation of genetic markers

Open reading frames (ORFs) selected for complementation of markers were cloned by using a forward primer 200 bases 5′ of the start codon, and a reversed primer 200 bases 3′ of the stop codon. The PCR was performed on genomic DNA of *A. niger* CBS 513.88, using pfu-polymerase (Promega). After the last cycle of amplification, Amplitaq (Applied Biosystems) and dATP were added, incubated at 72°C and 1 µl of the PCR mix was used in the pGEMT easy cloning kit (Promega). Of twenty different clones plasmid DNA was isolated and pooled. Transformations were performed essentially as described by Kusters-van Someren et al. (1991). A mixture of 1 µg of the pooled PCR-clones

Table 1 Strains used for the construction of diploids and two new marker strains

Diploid	Strain	Markers on III	Other markers
dp 1	N705	*cnxD9*	*metB10*
	N932	*lysA7 argD6*	*fwnA25; nicA1*
dp 2	N430	*argD6*	
	N720	*cnxD6*	*fwnA1; leuA1; nicA1*
dp 3	N893	*pyrA5 hisH8 bioA1*	*fwnA1; pheA1*
	N933	*argD6 cnxD6*	*nicA1*
dp 4	N934	*lysA7 cnxD9*	*fwnA25; nicA1*
	N935	*pyrA5 hisH8 bioA1*	*phenA1*
New marker	N936	*pyrA5 argD6 cnxD6*	*nicA1*
Strains	N937	*pyrA5 hisH8 bioA1 cnxD6*	*fwnA1; nicA1*

and 5 μg of an *ama1* containing plasmid (Verdoes et al. 1994b) was used for PEG-mediated transformation of 10^6 protoplasts. Transformants appearing were tested for their residual markers. The successful transformations were repeated using plasmid DNA from single clones.

Selection for mitotic crossing-over and segregation of diploids

Diploids were selected as described (Bos et al. 1989). Selection of *cnxD* homozygous diploids was done by growing single cell colonies of diploids on minimal medium (MM) with sodium nitrate as single nitrogen source and stab inoculation of these colonies on selective complete medium (CM) containing 150 mM $KClO_4$ and proper supplements for all markers present in the diploids. These diploids were analyzed by haploidization on CM plates with 0.88 mg/l D/L-benomyl followed by replica plating of the progeny to establish their fenotype.

Microarray data

The microarray data of all ORFs examined in this study are averaged data of days 3 and 5 expression levels using GeneChips for a glucose fed-batch culture of CBS 513.88 (Pel et al. 2007). The expression levels were normalized to an average of 100.

Results

Linking the genetic map to the physical map

A first goal was to identify genes complementing known genetic auxotrophic marker genes on Chromosome III and to link the position of these genes on the physical map with the genetic map. Candidate genes for the corresponding biosynthetic routes were selected from supercontigs 12 and 15, comprising Chromosome III specific contigs (Pel et al. 2007) by using the FunCat annotation (Ruepp et al. 2004) (Table 2). A second criterion for the selection of genes was presence of their transcripts in a GenChip hybridization experiment using RNA isolated from a glucose fed-batch fermentation (Pel et al. 2007). With two of the markers, *pyrA* and *areA*, already identified (Goosen et al. 1987; Lenouvel et al. 2001), 14 remaining candidates were cloned as genomic PCR clones. Using these clones, complementation was found for the genetic markers *bioA, bioB, lysA, hisH, argD*, and *cnxD*. In all cases, the auxotrophic mutant was complemented by only one of the candidate genes (Table 1). With 8 cloned markers we compared the physical map of chromosome III with the genetic map described before (Fig. 1, Debets et al. 1993). This comparison adds extra information

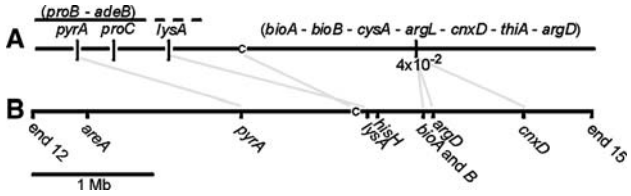

Fig. 1 Linking the genetic and physical map of chromosome III. **a** shows the genetic map of linkage group III as published by Debets et al. (1993). Markers with unknown gene order are shown in *parentheses* and/or above each other. **b** shows the physical map drawn to scale, consisting of supercontigs 12 and 15 of the *A. niger* genome sequence (Pel et al. 2007). The location of identified genetic markers is indicated. The centromere (*c*) is located in a gap of unknown size between the positions 0 of supercontig 12 and 15. For convenience the size of the gap is depicted as 0 bases

to the genetic map of chromosome III and is in accordance with the previously published genetic map (Debets et al. 1993), except for the position of the *lysA* marker.

Recombination of the genetic markers

The genetic map of Debets et al. (1993) gives limited information on the order of the markers because they were positioned individually, with the markers on the right arm at a crossing-over frequency of 4% from the centromere. This positioning of markers was established by selection for mitotic crossing-over in heterozygous diploids. The strains available at the beginning of our study contained only a few of the cloned genetic markers. Therefore, additional marker strains were constructed containing several auxotrophic mutations, which allowed the determination of genetic distances between these markers. Recombination of linked markers requires crossing-over, which is a rare event. Selection for crossing-over events is possible when using a recessive marker located more closely to the telomere than the other markers, as is the case for *cnxD* (Debets et al. 1990). The first recombination performed was the *cnxD9* mutation with *argD6* using diploid 1 (Table 1). From this heterozygous diploid, 15 independent homozygous *cnxD9* diploids were selected on chlorate. The colonies appearing on the chlorate plates were carefully checked for a sector-like growth, indicating that the homozygous diploids originated from independent crossover events. These diploids were analysed by haploidization on benomyl, and 14 of them appeared to have had a mitotic crossover between *lysA7* and *argD6*, one diploid had a crossover between *lysA7* and the centromere, but no crossover between *argD6* and *cnxD9* was found (Fig. 2). In a second recombination experiment using diploid 2 (Table 2) with *cnxD6* and *argD6,* we found one diploid with a crossover between *argD* and *cnxD*. The other 12 selected homozy-

Table 2 Complementation of genetic markers with selected genes and their position on supercontig 12 and 15

ORF name	Possible corresponding markers	Complementation found of mutation	No complementation of mutations	Position of the ORF (kb)
End of An12				2562
An12g08960	*areA*	*areAd*		2131
An12g07690	*lysA or lysE*		*lysA7*	1845
An12g03570	*pyrA*	*pyrA6*		907
An12g01280	*argD or argL*		*argD6 L25*	354
An12g01110	*cysA*		*cysA2*	319
An12g00320	*adeB E or G*		*adeB2 E8 G13*	76
Centromere				0
An15g00350	*lysA or lysE*	*lysA7*		94
An15g00610	*hisH*	*hisH8*		174
An15g01980	*bioA or bioB*	*bioB2*	*bioA1*	534
An15g01990	*bioA or bioB*		*bioA1 B2*	535
An15g02000	*bioA or bioB*	*bioA1*	*bioB2*	539
An15g02340	*argD or argL*		*argD6 L25*	612
An15g02360	*argD or argL*	*argD6*	*argL25*	618
An15g05170	*cysA*		*cysA2*	1220
An15g05720	*cnxD*	*cnxD6*		1334
End of An15				1877

gous *cnxD6* diploids resulted from crossovers between the centromere and *argD6* (Fig. 2). Of the first crossed-over diploid N933 was isolated, which was subsequently used for the construction of diploid 3.

Delimiting the recombination hotspot

Apparently, in *A. niger* there is a preference for mitotic crossover in the genomic region between *lysA* and *argD* compared to the region between *argD* and *cnxD* (Fig. 2). First mentioned region overlaps the region between *lysA* and *bioA* where the previously reported recombinations were found (Bos et al. 1988). To further examine the distribution of mitotic crossovers we analysed 26 homozygous *cnxD6* diploids selected from diploid number 3 and 18 homozygous *cnxD9* diploids selected from diploid number 4. In the first case, 25 crossovers occurred between the centromere and *hisH8* and only 1 between *argD6* and *cnxD6*. In the latter case, only 1 crossover occurred between the centromere and *lysA7*, 10 between *lysA7* and *hisH8*, 2 between *hisH8* and *bioA1* and 5 between *bioA1* and *cnxD9* (Fig. 2). This clearly shows that there is a recombination hotspot in the 80 kb region between *hisH* and *lysA*.

Analysis of the recombination hotspot

Possibly the region between *hisH* and *lysA* contains genetic elements that increase crossing-over. We searched for microsattellite repeats (Majewski and Ott 2000) and polyA stretches, using the pattern search on the Biomax server

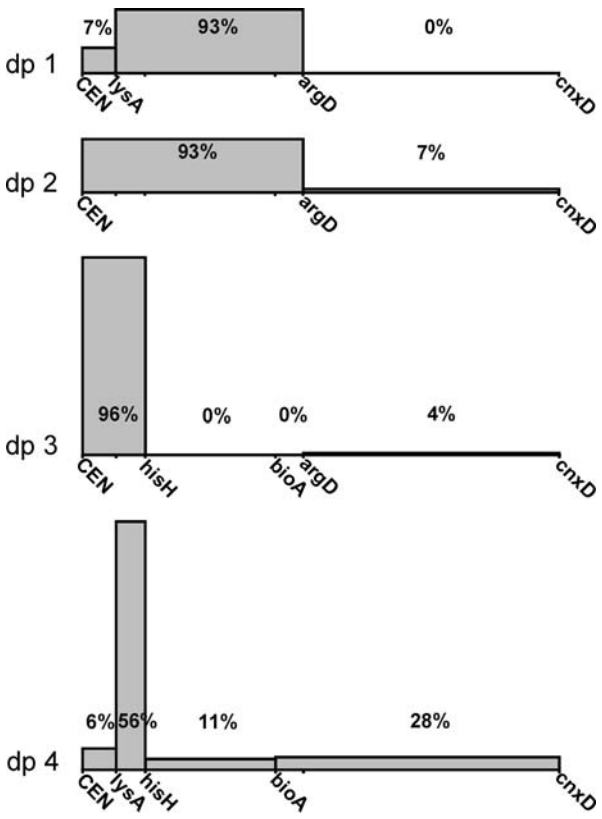

Fig. 2 Frequency of crossing-over between centromere and *cnxD*. In four diploids, homozygous *cnxD6/9* diploids were selected on chlorate. The regions of crossover are delimited by the markers used in the four diploids and are positioned between the centromere (CEN) and *cnxD*. The frequency of the crossing-over in a region correlates with the surface of the *box* depicted above that region

(Frishman et al. 2003). Furthermore, the expression profiles of all ORFs on supercontig 15 were examined, using the microarray data published by Pel et al. (2007) (Fig. 3a, b) and the Genome Atlas of supercontig 15, kindly provided by Dr. D. Ussery using previously developed tools (Skovgaard et al. 2002; Wanchanthuek et al. 2006) (Fig. 3c, d). We found a short polyA stretch and three GA stretches and at the telomeric side of the *hisH* gene a CT repeat. Looking at expression across supercontig 15 on a scale of 0–3,000, there is no apparent abnormality near the mitotic recombination hotspot (Fig. 3a). There is a large region from 990 to 1,240 kb where gene expression seems to be low. With the expression plotted on a scale from 0 to 100, it becomes clear that within the mitotic recombination hotspot, particularly around *hisH*, the lowest transcribed ORFs have a higher transcription compared to the lowest transcribed ORFs outside this region (Fig. 3b). Another feature deviating along the complete *lysA–hisH* region is a more negative stacking energy, indicating a more stabilized region (Skovgaard et al. 2002) and a low AT percentage. The position preference, a measure for flexibility (Baldi et al. 1996) is only slightly higher near *hisH*. There are a few simple repeats in the *lysA–hisH* region and there are multiple purine or pyrimidine stretches of minimal 10 bases ((Y)10 vs. (R)10) around *hisH*, coinciding with an elevated GC skew (G-C)/(G + C) and AT (A-T)/(A + T) skew. A zoom-in of the *lysA–hisH* region is shown in Fig. 3d.

Occurrence of purine stretches in supercontig 15

Purine stretches inside ORFs are known to block transcription via the formation of an intramolecular triple helix

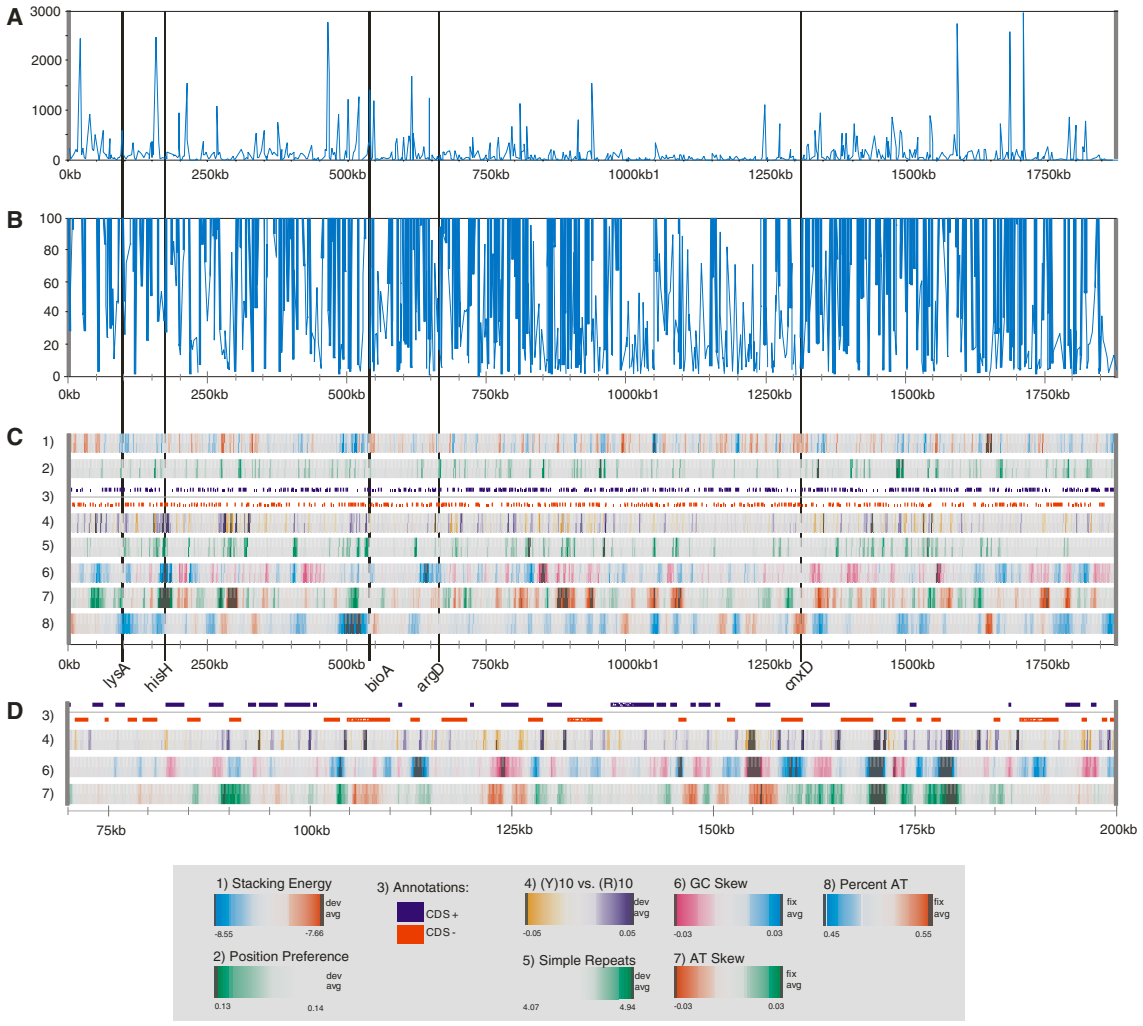

Fig. 3 Genome atlas and expression profile of supercontig 15. Expression of all genes on supercontig 15 are plotted in two scales **a** and **b**. The genome atlas of supercontig 15 according to **c** shows repeats, structural parameters and parameters directly related to the base composition. The region studied for mitotic recombination is located between the centromere (*left* of 0 kb) and *cnxD* (1334 kb). The recombination hotspot is located between *lysA* (94 kb) and *hisH* (174 kb)

(Bidichandani et al. 1998; Grabczyk and Fishman 1995) while purine stretches were also reported to occur more often in fungal genomes than could be expected from random sequences (Ussery et al. 2002). As the genome atlas shows the occurrence of purine stretches to be different in the genomic region of interest, we had a closer look at them. Inside the coding DNA located in the region between *lysA* and *hisH*, the largest purine stretches are 17 and 16 bases, respectively. Outside the coding DNA there are 6 purine stretches of 32–65 bases long, and 2.7 kb at the centromeric side of *hisH*, an 80 bp purine stretch containing only two pyrimidines.

Discussion

To link the genetic map to the physical map, thirteen ORFs were selected to possibly complement eleven markers. Six of those genes indeed complemented a corresponding marker, which is indicative for the high quality of the genome annotation. Six of these markers and two already cloned markers *areA* and *pyrA*, were positioned on the genetic map by Debets et al. (1993; see also Fig. 1). The position found for *lysA* on the right arm is not in agreement with the expected position left of the centromere. The experimental evidence for the left arm position is mainly provided by selection for mitotic crossing-over on fluoroorotic acid (FOA) in a diploid heterozygous for *pyrA5* and *lysA7* (F. Debets, personal communication). FOA is very mutagenic compared to chlorate and possibly induced additional crossovers that led to a false positioning of *lysA*. In our results with *cnxD* selected crossovers in diploids 1 and 3, we found further evidence for *lysA* to be located on the right arm (Fig. 2). The resulting physical map can be seen as an improved genetic map because more markers are placed in the correct physical order. The genetic distance of these markers, however, is still unclear.

A series of mitotic recombinations to combine multiple genetic markers into one single master strain proved much more difficult than expected because of the presence of a mitotic recombination hotspot in the region between *lysA* and *hisH* (Fig. 2). There are several examples of increased recombination in genomic regions, both during mitosis and meiosis. Meiotic recombination hotspots can be caused by increased double-strand breaks in defined genomic regions (Nishant and Rao 2006). In yeast, mitotic recombination in general occurs more often near the centromere (Minet et al. 1980) and a mitotic recombination hotspot is also proposed to act via double-strand breaks (Neitz and Carbon 1987), while *HotI* stimulates mitotic recombination in adjacent sequences (Stewart and Roeder 1989). In *A. nidulans*, there is a position dependent difference between meiotic recombination and mitotic recombination. Using sexual crosses, genetic distances near the centromeric region appear larger than the physical distances (Aleksenko et al. 2001; Espeso et al. 2005), while the opposite is found when using mitotic recombination (Käfer 1977). Although there seems to be a preference for mitotic crossing-over near the centromere, we conclude that the 80 kb region between *lysA* and *hisH* contains a true mitotic recombination hotspot, because most mitotic crossover events occur in that region and not in the 94 kb centromere-*lysA* region (Fig. 2).

The expression graphs and the genome atlas (Fig. 3) visualize some interesting features of the mitotic recombination hotspot. Over the whole *lysA–hisH* region there is a high basic transcription, indicating an open chromatin structure (Fig. 3b). A high AT percentage compared to the rest of the supercontig causes an increased stacking energy, which indicates a more stable helix (Ornstein et al. 1978). Around *hisH*, both GC-skew and AT-skew are higher, coinciding with purine stretches (Ussery et al. 2002). In some plants and fungi, an elevated GC-skew near the transcription start sites of highly transcribed genes was found (Fujimori et al. 2005). As mentioned before, purine stretches can have a drastic effect on transcription. The *lysA–hisH* region contains purine stretches outside coding DNA, whereas inside the ORFs no apparent purine stretches are found. Additionally, the region of low transcription from 990 to 1,240 kb contains very few purine stretches (Fig. 3) and is situated between the markers *argD* and *cnxD*, which show very little recombination. Ussery et al. (2002) reported that in microbial eukaryotic chromosomes, the purine tracks occur more often than would be expected from random sequences, and are localized mainly in non-coding regions. Also, a relation between mitotic recombination frequencies and transcription levels (Saxe et al. 2000; Aguilera 2002; Grewal and Elgin 2002) may be relevant in the *lysA–hisH* region.

All together, it is not clear whether the deviating physical properties of the DNA cause the mitotic recombination hotspot or influence the level of transcription, and if so, which of them is most important. However, the connection between noted properties could be through elevated basic transcription, causing or caused by an open chromatin structure that could lead to an increased mitotic recombination in the *lysA–hisH* region. Especially the presence of purine stretches outside coding DNA, and the absence of purine stretches inside coding DNA seem to correlate with basic transcription and mitotic recombination. However, we cannot exclude that the mitotic recombination hotspot could also be caused by a yet unidentified genetic element.

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